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PRINCIPAL INVESTIGATOR: F. Sessions Cole, M.D.

CONTRACTING ORGANIZATION: Washington University
Saint Louis, Missouri 63110

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13. ABSTRACT *(Maximum 200 words)*

We studied developmental changes in glucose transporter targeting in mammary gland, sought novel proteins affecting GLUT1 targeting, and studied glucose transport in breast cancer cells. Most significant conclusions are:

1. In normal CIT3 mammary epithelial cells, GLUT1 colocalizes with Golgi markers b-COP and a-mannosidase but not with the trans-Golgi marker Bodipy TR-ceramide.
2. There are no higher molecular weight isoforms of GLUT1.
3. Glycosylation plays no role in GLUT1 targeting to Golgi.
4. There is no evidence that lactogenic hormones stimulate expression of a novel glucose transporter.
5. Changes in Golgi markers with forced weaning suggest that changes in GLUT1 targeting during that time may reflect a dynamic reorganization process affecting all Golgi constituents.
6. GLUT1-EBFP fusion protein offers the opportunity to study transporter targeting in living cells.
7. Golgi GLUT1 purified under non-denaturing conditions has an apparent molecular weight of 130 kD, suggesting that it may be associated with a protein of 70-90 kD.
8. MDA231 cells exhibit very high rates of glucose transport but do not appear to utilize GLUT1 for this purpose, suggesting expression of a novel transporter or "oncotransporter."; these cells sequester GLUT1 in an atypical-appearing intracellular compartment whether or not prolactin and hydrocortisone are present.

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Peter M Haas MD FACP 10/7/98

PI - Signature Date

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Introduction-

Prologue-

As of July 1, 1997, I became an Assistant Professor of Pediatrics at the Baylor College of Medicine in Houston Texas. I am affiliated with the Section of Neonatology and with Texas Children's Hospital and my lab is in the U.S.D.A./A.R.S. Children's Nutrition Research Center.

Note that, since DOD policy did not allow the grant to be transferred from Washington University to Baylor College of Medicine, F. Sessions Cole, M.D. graciously agreed to serve as Principal Investigator and to subcontract the work to me at Baylor College of Medicine. Therefore, I am technically submitting this report on his behalf.

a. the subject of the research

Glucose is critical to mammary epithelial cells not only because it serves as a fuel and as a building block for glycoproteins and glycolipids, but also because of its role as the major substrate for the synthesis of lactose and lipid, which together contribute 80-90% of the calories in human milk. Lactose is the major carbohydrate constituent of human milk and the major determinant of its osmolarity, and therefore, of milk production. Synthesis of lactose is carried out exclusively within the Golgi apparatus of mammary epithelial cells, in a reaction catalyzed by galactosyltransferase complexed to the tissue-specific protein alpha-lactalbumin(Strous, 1986). Lipid components of human milk, which are also primarily derived from glucose, provide about one-half of the caloric content of milk. Thus, regulation of glucose uptake in mammary epithelia must account for two very different states, the quiescient state, with a relatively small demand for glucose, and the lactating state, with an extraordinary demand for glucose to fuel the high metabolic rate of the epithelial cells themselves as well as to provide substrate for the synthesis of milk to sustain the young.

Breast cancer cells also exhibit an increased demand for glucose, as reviewed below. The molecular mechanisms by which the enhanced transport of this vital nutrient into tumor cells is accomplished require further investigation. Elucidation of the molecular mechanisms by which the mammary epithelia achieves the adaptations in glucose transport needed for lactation, and the examination of their possible dysregulation in neoplastic mammary epithelium, form the central goals of this proposal.

b. the background of previous work

There are two mechanisms for glucose transport into cells. For most cells, the sole mechanism is the passive diffusion of glucose into cells, facilitated by the five isoforms of the glucose transporter family(Burant, et al., 1992, Mueckler, 1994). These are designated GLUT1-GLUT4, and GLUT7, in the order in which they were cloned. (GLUT5 is actually a fructose transporter(Burant, et al., 1992), and GLUT6 is a

pseudogene related to GLUT5(Kayano, et al., 1990)). These isoforms exhibit distinct regulatory properties, tissue distributions, and kinetics. However, they are all integral membrane proteins containing approximately 500 amino acids. Hydropathy plots based on amino acid sequences predicted from cDNA sequence suggest that all share a common topology, which includes cytoplasmic N- and C-termini, twelve membrane spanning domains, one exofacial loop which contains an N-linked glycosylation site, and one approximately 65 amino acid intracellular loop midway through the transporter. There is a striking degree of homology among these isoforms, which are 50-65% identical in their amino acid sequence. GLUT1 is also known as the endogenous glucose transporter because of its nearly ubiquitous tissue distribution. It is important in basal glucose uptake and is usually found primarily in the plasma membrane. It is the only glucose transporter isoform convincingly shown to be expressed in mammary epithelia. In the specialized setting of intestinal(Hwang, et al., 1991) and renal(Miller, et al., 1992, Pajor, 1994) epithelia, and possibly in pulmonary epithelia(Kemp and Boyd, 1992), glucose is also taken up by active transport across specialized membrane domains by tissue-specific isoforms of the sodium, glucose co-transporter(Hediger, et al., 1987). A possible role of this protein in mammary gland glucose transport during lactation has recently been suggested(Shennan and Beechey, 1995).

Mammary gland is unique in its requirement for free glucose within the Golgi, the site of lactose synthesis from glucose and UDP-galactose. The substrates for glycosylation of proteins within the Golgi, which occurs in many cell types, are nucleotide sugars, not free sugars. Wilde and Kuhn measured glucose uptake into rat mammary acini at different glucose concentrations, and directly measured intracellular glucose concentration, to conclude that glucose transport is rate-limiting for lactose synthesis(Wilde and Kuhn, 1981). Madon et al. measured cytochalsin B binding of fractionated rat mammary gland and found that the GLUT1 glucose transporter accounted for only about one-half of the cytochalasin B binding sites of Golgi, strongly suggesting that a unique transporter resides in the Golgi of lactating mammary gland(Madon, et al., 1990). This proposal aims to extend our understanding of glucose transport within lactating mammary gland by identifying and characterizing the molecular species responsible for glucose transport within the mammary epithelial cell and by exploring their developmental and hormonal regulation. This forms a prerequisite for understanding glucose transport in breast cancer.

Seven decades ago, Warburg(Warburg, 1923) appreciated that tumor cells show high rates of glucose uptake, glucose metabolism, and respiration(Hatanaka, 1974, Merrall, et al., 1993). Several lines of evidence suggest the value of a comprehensive understanding of glucose transport in mammary gland in the context of breast cancer. Brown et al.(Brown and Wahl, 1993) showed that higher expression of the glucose transporter GLUT1 by breast cancer cells compared with the healthy breast tissue is common. Several groups have recently shown that the glucose analog 18-F-fluoro-2-deoxyglucose can be used to detect and stage breast cancer(Wahl, et al., 1991, Tse, et al., 1992, Nieweg, et al., 1993, Adler, et al., 1993, Crowe, et al., 1994), suggesting that an

abnormally high uptake of glucose is a consistent finding in breast cancer. The MCF-7 line of breast cancer cells was established over twenty years ago(Soule, et al., 1973) and has been characterized in literally hundreds of studies since then. Inhibition of glycolysis in MCF-7 breast cancer cells by extracellular AMP markedly inhibited cell proliferation(Hugo, et al., 1992). Elegant NMR studies have recently shown that, in MCF-7 human breast cancer cells, tamoxifen inhibits glucose consumption and lactate production by 50%, compared to estrogen-treated cells, and that estrogen rescue of tamoxifen treated cells was associated with a rapid increase in glucose consumption(Furman, et al., 1992, Neeman and Degani, 1989). Glucose-6-phosphate dehydrogenase, a key enzyme of glucose metabolism, is strikingly elevated in mammary epithelial cells from patients with breast cancer compared to those with benign breast disease(Barron, et al., 1991). Its activity was also significantly increased in morphologically normal tissue from cancer-containing breasts when compared to breasts with no known cancer, suggesting the possibility that metabolic abnormalities precede morphological changes in breast carcinogenesis(McDermott, et al., 1990). GLUT1 is the major glucose transporter isoform expressed in mammary epithelial cells. Importantly, GLUT1 is also the only known glucose transporter isoform whose gene is activated at the level of transcription in cells transformed by oncogenes such as *fps* , *src* , and *ras* (Birnbaum, et al., 1987, Flier, et al., 1987). Since this response is direct, the GLUT1 gene is an immediate early gene. Of special interest with respect to breast cancer is that the *neu* oncogene induces synthesis of GLUT1 mRNA and increases glucose uptake three-fold in fibroblasts(Sistonen, et al., 1989). Several groups have reported that between 18.8% and 67% of patients with breast cancer have elevated serum *neu* protein levels or amplification of HER-2/*neu*.(Kath, et al., 1993, Charpin, et al., 1993, Descotes, et al., 1993, Bacus, et al., 1992). Induction of GLUT1 mRNA synthesis also occurs in cells after addition of serum, peptide growth factors, and agents which increase intracellular cAMP concentration(Hiraki, et al., 1989). The two enhancer elements responsible for the responsiveness of the GLUT1 gene to growth factors and oncogenes have been characterized(Muramiki, et al., 1992).

What might the biochemistry and molecular cell biology of glucose transport in lactating mammary gland teach us about breast cancer? One fundamental answer is that, to understand the abnormal state of a cell, one must first understand its normal function and development. A more concrete rationale is suggested by the observation that high rates of glucose uptake and high levels of GLUT1 characterize breast tumors, as noted above. Furthermore, certain proteins important in lactation are also expressed in neoplastic breast tissue but not in normal, quiescent, breast tissue. Serum human alpha-lactalbumin, the mammary-specific protein cofactor that combines with galactosyltransferase to form the complex lactose synthetase, has been proposed as a marker for breast cancer(Thean and Toh, 1990). Similarly, a milk fat globule protein is highly expressed in human breast tumors(Hilkens, et al., 1986, Larocca, et al., 1991). There is also a higher molecular weight glycoprotein detectable in milk and breast carcinomas(Sekine, et al., 1985). None of these are expressed in non-lactating, non-neoplastic mammary gland.

c. the purpose and scope of the present work

In contrast to the transfer of lactose into milk across apical membrane, which occurs by vesicle fusion and is not carrier-mediated, glucose must be transported across at least three distinct cellular membranes of lactating mammary epithelial cells. Specifically, glucose must be transported from the blood across the basal plasma membrane to the mammary gland cytoplasm, from the cytoplasm across the Golgi membrane to the Golgi, where lactose synthesis occurs(Kuhn and White, 1975), and from the cytoplasm across the apical membrane to milk. No known isoforms of the glucose transporter family are known to reside primarily in the Golgi. The regulation of glucose transport must take into account the difference in requirements of the quiescent and the lactating gland. Therefore, the specific hypotheses to be tested are:

1. Glucose transport into mammary epithelial cells is subject to a high degree of regulation.

GLUT1 and any other transporter isoforms or other novel proteins identified by pursuing the first specific aim are likely to exhibit developmental and hormonal regulation. The activities of key enzymes of lactation, such as acetyl-CoA-carboxylase, fatty acid synthetase, galactosyltransferase, phosphofructokinase, and isocitrate dehydrogenase, among others, expressed per mg DNA, rise several-fold as lactation commences(Wilde, et al., 1986). It is reasonable to expect coordinate regulation of glucose transporters. Given the crucial importance of successful lactation to continued survival of the species, and given the limited evidence that already exists, I expected to confirm that glucose transport into lactating mammary gland is, in fact, exquisitely regulated, and proposed to establish the molecular mechanisms of that regulation.

2. Abnormal glucose transport in mammary epithelial cells (i) is associated with abnormal cell growth, and (ii) may facilitate abnormal cell growth.

Only by comprehensively characterizing glucose transport in normal quiescent and lactating mammary gland will we obtain the data needed to understand the significance of a possible role for altered glucose transport in breast cancer. As noted above, there is substantial evidence linking glucose uptake and metabolism with cancer. I anticipate that the association between glucose metabolism and breast cancer will be confirmed. Using techniques of molecular cell biology, I will be able to test whether specific perturbations of glucose transport, including those which might be found to be associated with cancer, can of themselves confer an abnormal phenotype. I also intend to alter glucose transporter targeting in tumor cells to reduce the amount at the cell surface available for glucose uptake; this will test the hypothesis that abnormally high glucose transport is important to support tumor cell growth. There are currently no data upon which to base a prediction of the results of this experiment, although there is the precedent that the *v-sis* oncoprotein loses transforming activity when targeted to

the early Golgi complex(Hart, et al., 1994). Successful reduction of glucose transport into tumor cells might offer new therapeutic possibilities.

In order to test these hypotheses, the following specific aims were chosen:

1. Description of the developmental and hormonal regulation of glucose transport in mammary gland.

GLUT1 has been identified in total plasma membrane and Golgi fractions of lactating rat mammary gland. As described above, a single method, subcellular fractionation by differential centrifugation, was used to examine subcellular distribution, and the possibility of contamination with other cellular compartments can not be excluded. I proposed to use several methods to determine the distribution of GLUT1 in lactating rat mammary gland, in primary epithelial cells of this gland in culture(Barcellos-Hoff, 1989), and in CIT_3 and Comma 1-D cells, established mouse mammary epithelial cell lines(Danielson, et al., 1984). Note that I am presenting data from mouse rather than rat. I have chosen to focus on mouse for several reasons. First, the established cell lines are murine. Secondly, the mammary gland transplantation technique I will soon be applying has been used in mice but not rats. Thirdly, normal mouse data would be needed to interpret data from any transgenic or knockout mice I might generate in work beyond the scope of this porposal. Dr. Peggy Neville of the University of Colorado School of Medicine has provided me with CIT_3 cells, which she has selected from Comma-1-D cells for their ability to grow well on filters, form tight junctions, and exhibit polarized transport (personal communication). Mice will be studied at four different stages, non-pregnant-non-lactating, late-pregnant, lactating, and involuting. Details of certain procedures, namely cell culture, [^{3}H]-2-deoxyglucose uptake assay, transfection, Western blot analysis, confocal immunofluorescence microscopy, iodixanol density gradient analysis, and immunogold labeling and electron microscopy, are found in my previous publications(Haney, et al., 1991, Haney, et al., 1995).

The subcellular distribution of GLUT1 will be examined in polarized cells. Polarity will be established by growing cells on tissue culture inserts(Parry, et al., 1987). To test the possible role of extracellular matrix in mammary epithelial glucose transport, these inserts can be coated with basement membrane matrices such as those derived from EHS tumors, which have been shown to influence gene expression and differentiated functions(Aggeler, et al., 1991, Lin, et al., 1995). The initial approach to subcellular distribution will be using confocal immunofluorescent microscopy, which results in sensitive and specific staining of GLUT1 protein. Cells will be exposed to specific affinity-purified antibodies, and then to appropriate fluorescent secondary antibodies. Through careful selection of antibody concentrations to be used, the possibility of non-specific staining is minimized, then ruled out using appropriate controls. In this way, it is possible to localize a specific protein to basolateral membrane, apical membrane, Golgi membrane, or another intracellular compartment. An important limitation of this

method is that it does not yield quantitative data. Basolateral membrane and apical membrane will be distinguishable(Sjaastad, et al., 1993) because of the polarity of cells grown on filters and because of the nature of confocal microscopy, which visualizes only one section of a cell at a time. Apical or basal membrane markers will be visualized with a second fluorescent antibody. Golgi can be stained using antibodies specific for the Golgi markers alpha-mannosidases I and II(Velasco, et al., 1993, Antony, et al., 1992). Brefeldin A is a fungal metabolite that causes disassembly of Golgi(Klausner, et al., 1992); failure of GLUT1 to alter its subcellular distribution after treatment with Brefeldin A would suggest it was not a Golgi resident(Berger, et al., 1993). Confocal immunofluorescent microscopy will also be applied to sections from lactating and non-lactating mouse mammary gland to determine whether the subcellular localization of GLUT1 changes with differentiation.

Immunogold labeling and electron microscopy have been useful tools in defining the intracellular compartments in which glucose transporters are sequestered and in quantitating their subcellular distribution(Haney, et al., 1995, Slot, et al., 1991, Slot, et al., 1991). The distribution of endogenous GLUT1 under different conditions, such as quiescence, lactation, and neoplasia, and the distribution of heterologous transporters or other proteins of interest in experiments described below, will be studied using this method.

Subcellular distribution will be confirmed by subcellular fractionation using iodixanol density gradient centrifugation. The distibution of GLUT1 across the gradient will be directly compared with the distribution of the plasma membrane marker, 5'-nucleotidase, and the Golgi marker, alpha-mannosidase I. Protocols exist for separation of apical and basolateral plasma membrane fractions in other polarized epithelial cells(Mircheff, 1989). I intend to adapt these to mammary epithelium as well.

The possibility that other glucose transporter isoforms might be found in mammary gland has not been rigorously excluded. The simplest way to do so is to attempt to detect the mRNA for the other known transporters, GLUT2, GLUT3, GLUT4, GLUT7, and the sodium, glucose cotransporter, by Northern blotting rat mammary gland poly(A)$^+$ RNA with cDNA for each transporter. As described above, mice will be studied at four different stages, non-pregnant-non-lactating, late-pregnant, lactating, and involuting. Similar studies will be performed using neoplastic human breast tissue, and established cell lines representing both non-neoplastic (CIT_3) and neoplastic (MCF-7) states. Results will be confirmed by performing Western blots. The expression of these isoforms will also be studied in cells isolated from human milk(Lindquist, et al., 1994), and in cells isolated from reduction mammoplasty specimens. Dr. Mike Mueckler of the Washington University School of Medicine has supplied me with cDNAs for all known isoforms. If another isoform is detected, its abundance, subcellular distribution, and activity will be studied as described above for GLUT1. Dr. Mueckler has also supplied me with the available specific antibodies for all isoforms.

Established cell lines as well as cells in primary culture will be exposed to factors known to influence mammary epithelial cells, such as prolactin, growth hormone, and insulin-like growth factors(Peters and Rillema, 1992, Flint, et al., 1992, Barber, et al., 1992), among others, to determine whether there is any direct effect on glucose transport. The amounts of glucose transporters can be increased by DNA-mediated transfection of expression vectors which result in their synthesis at high levels; an increase in lactose synthesis in cells expressing supraphysiologic levels of a specific glucose transporter would confirm that glucose transport limits lactose synthesis and milk production.

2. Examination of a possible association between abnormal glucose transport and the neoplastic phenotype.

Glucose transport in MCF-7 breast cancer cells, which exhibit polarized expression of membrane glycoproteins(Zou, et al., 1989), will be characterized by the methods described above. This will include comparisons of hormonal responsiveness of glucose transport. This observational study will describe differences between two established cell lines, the CIT_3 and MCF-7 lines, but differential expression of transporters or other regulators between the two lines, while suggestive, cannot prove the importance of a given protein.

Brown et al.(Brown and Wahl, 1993) examined twelve breast tumors and showed by immunohistochemistry that higher expression of the glucose transporter GLUT1 by breast cancer cells compared with the healthy breast tissue is common. However, these investigators did not quantitatively assess glucose transporter targeting. I intend to carry out these studies quantitatively, at the level of mRNA as well as protein, in order to understand the magnitude of the changes in glucose transport seen in neoplasia. Tom Wheeler, M.D., of the Department of Pathology at Baylor College of Medicine, is also collaborating with me in this aspect of the study; the up to 20 breast cancer specimens per year referred to above will also be studied to determine patterns of glucose transporter expression in breast cancer. I anticipate studying at least 20 specimens before drawing conclusions.

The observational approach outlined in the previous paragraph can not distinguish whether changes in glucose transporter expression, which are, after all, likely to be observed, are central and necessary phenomena, or simply epiphenomena. The relationship between expression of a specific glucose transporter and the neoplastic phenotype will therefore be directly tested by stable transfection as described above. Normal mammary epithelial cells will be stably transfected with expression vectors containing the non-inducible CMV promoter, and will express the heterologous transporter constitutively. This will directly test the link between the transporter in question and changes in phenotype, including altered transport properties, glucose utilization, synthesis of milk components, growth characteristics, and cellular morphology. Overexpression in neoplastic cells of factors which might alter glucose

transporter activity will directly test the importance of elevated glucose transport activity in contributing to tumor growth. Glucose transporter levels can also be reduced up to 80% using antisense RNA methods(Valera, et al., 1994), providing another avenue for determining the significance of GLUT1 overexpression for the neoplastic mammary cell phenotype. An expression vector based on the mouse mammary tumor virus promoter, which is active in mammary gland(Gunsburg and Salmons, 1986), confers highly inducible synthesis of heterologous proteins in epithelial cells(Hirt, et al., 1992). This will be useful in ruling out any effects of constitutive expression on membrane trafficking or differentiation, and in establishing more firmly the link between expression of heterologous protein and changes in phenotype.

An elegant method for reconstitution of mouse mammary gland from mammary epithelial cells(DeOme, et al., 1959, Medina, 1973) has been adapted to cells genetically altered in vitro(Edwards, et al., 1992) in order to study physiological and morphological correlates of oncogene expression. Normal mammary epithelial cells are isolated from one mouse and briefly put into primary culture, where a gene is introduced by retroviral infection; the cells are then transplanted into the mammary fat pad of a mouse from which the endogenous epithelium has been removed. The transplanted cells grow to reconstitute a "tree" of glandular epithelium. Transplants carrying the Wnt-1 oncogene grew in a hyperplastic pattern, showing abundant fine side-branches, but without development of alveoli. The same authors also showed that expression of the neu/erbB-2 oncogene induced epithelial abnormalities similar to human atypical hyperplasia and sclerosing adenosis(Bradbury, et al., 1993). This will be a useful method to study specifically in vivo the consequences on cellular organization and function of the overexpression of transporters or other unique factors identified in the course of this study. I will also explore the possible use of this system to understand the impact of these genetic alterations on lactose synthesis in vivo. The results of experiments with reconstituted mammary glands will serve to validate, or to question the significance of, findings from established cell lines or tumors.

Body

Experimental methods and procedures

Subcellular fractionation- (Haney, et al., 1991)- Glands were removed and homogenized in PBS (5 cc/g) with 1 mM EDTA as described above. Centrifugation for 10 min at 3000 g produced a pellet, which was resuspended and centrifuged again at 3000 g. This 3000 g pellet is the nuclear pellet. The combined supernatants of the 3000 g centrifugations were centrifuged for 10 min at 17000 g. This 17000 g pellet is the light mitochondrial pellet and is enriched in Golgi. This pellet was subjected to a self-generating iodixanol density gradient (10%-37%) by combining with a 15% iodixanol solution and centrifuging for 3 h at 180000 g. Fractions were collected using a Labconco Auto-Densiflow collector. Aliquots containing 20 µg of protein were subjected to Western blotting as described above. Prior to electrophoresis, in one experiment PNGase F was used for deglycosylation of glycoproteins, including GLUT1. 10ug protein samples were added to 2x sodium phosphate Buffer (pH 7.4), with 6 units/ml N-glycosidase F (Boehringer Mannheim), and incubated at 37°C overnight. 10% glycerol was used as a negative control.

Western blots- (Haney, et al., 1991; Haney and Mueckler, 1994)- Homogenates were prepared as described above and solubilized in 1%SDS. Samples were subjected to SDS-polyacrylamide gel electrophoresis, with 20 µg protein per lane. Samples were not be boiled before loading, since this distorts electrophoresis of membrane proteins. Purified human erythrocyte GLUT1 supplied by Dr. Mueckler served as standard. Proteins were transferred to nitrocellulose. Membranes were treated with Blotto for 30 min and exposed to primary antibody for 1 h. Primary antibody was a 1:1000 dilution of the highly specific, well-characterized F350, directed against the final 16 amino acids of the C-terminal cytoplasmic tail of GLUT1. Other antibodies from commercial sources were used for detection of marker proteins. Blots were washed three times for ten minutes each with PBS containing 1% SDS. Secondary antibody was horseradish peroxidase-antirabbit IgG, and signal was developed by the Amersham ECL protocol. Relative protein levels were determined by densitometry. Results shown are representative of two to three independent studies per timepoint.

Immunofluorescent microscopy- (Haney, et al., 1991; Haney and Mueckler, 1994)- Frozen tissue sections were prepared. This is the gentlest method available and preserves cell structure and antigens. A 0.5 cm x 0.5 cm x0.5 cm piece of gland was dissected and frozen gradually in Lipshaw Number 1 solution, and stored at -70°C. Using a cryostat, 5-10 µm sections were prepared. Sections were air-dried, dipped in paraformaldehyde for 2 min, washed in PBS, and placed in 1%NP40, PBS, for 5 min, then rinsed several times in PBS. Sections were exposed to peptide-affinity purified GLUT1 antibody, or to other antibodies from commercial sources were used for detection of marker proteins,

at a concentration of up to 5 µg/ml overnight at 4°C in a humidified chamber. Through careful selection of antibody concentrations to be used, the possibility of non-specific staining was minimized, then ruled out using appropriate controls, include antibody preabsorbed with the antigenic peptide. Sections were washed three times for 5 min each in PBS with 1% Triton X-100. Secondary antibody was FITC-labeled anti-rabbit IgG $F(ab)_2$ in PBS, 0.1% horse serum, applied at the recommended concentrations (Organon Teknika) for 1 h at room temperature. Sections were rinsed three times for 5 min each in PBS with 1% Triton X-100. One drop of Vectashield anti-photobleaching agent was applied, then coverslips were placed. Specimens were viewed using a Olympus ix70 microscope equipped for fluorescence, or with a Molecular Dynamics confocal workstation at the Baylor College of Medicine microscopy core. Localization of signal to basolateral membrane, apical membrane, Golgi membrane, and/or other intracellular compartments was assigned by surveys of low- and high-power fields.

Blue Native PAGE- (Schagger and von Jagow, 1991)- Samples were processed as in the subcellular fractionation. The first fraction from a 17000g pellet iodixanol density gradient was collected and solubilized with 1.25% dodecyl maltoside in the presence of 6-aminocaproic acid. After a 30-minute 13000 rpm centrifugation at 4°C, the supernatant was supplemented with Coomassie Brilliant Blue G. 100 ul of the supplemented protein solution was added to Blue Native PAGE non-denaturing gels and run for 3h at 215V at room temperature in cathode buffer A. After about one-third of the whole run, the electrophoresis was continued with a similar cathode buffer containing no dye (50mM Tricine, 15mM Bistris, pH7.0). After the run was completed, the gels were transferred to nitrocellulose membranes at 20V/4°C overnight. Western Blotting was performed as described above.

GLUT1-EBFP fusion protein- pEBFP-N1 was obtained from CLONTECH (Palo Alto, CA) and standard molecular biological techniques (Maniatis, 1989) were used to subclone the GLUT1 cDNA, kindly supplied by Mike Mueckler (Washington Univ., St. Louis). Transfections were carried out using liposomes (Lipofectin, GIBCO). Cells were selected in G418 at 0.5 mg/ml.

Summary of previous reports

In work funded by this grant and reported in previous annual reports, double-label immunofluorescence and subcellular fractionation by density gradient centrifugation were used to demonstrate that GLUT1 is localized, both in vitro and in vivo, in the Golgi in response to the hormonal milieu of lactation. Northern and Western blots for GLUT1 and GLUT5 indicated that the developmental regulation of glucose transporters was isoform-specific, and a rapid decline in GLUT1 levels at weaning was linked to changes in the translational efficiency or increased GLUT1 degradation.

During the previous reporting period, I focused on the weaning period, since it offers the opportunity to examine whether transporter targeting is altered at a point where it is imperative that milk production be rapidly curtailed. Failure to observe changes in glucose transporter targeting would have suggested that this is not an important component of the mechanism which regulates milk production. To examine the regulation of GLUT1 targeting during lactation, mouse pups were prematurely weaned at 18 days of age, the peak of milk production. This tested the hypotheses that GLUT1 is targeted to Golgi during lactation, and that its regulation during weaning would be consistent with an important role in the regulation of lactose synthesis. Subcellular fractionation and density gradient centrifugation were employed to isolate a Golgi-enriched fraction of mammary gland. Enrichment in Golgi but not plasma membrane was verified using galactosyl transferase as a Golgi marker and 5'-nucleotidase as a plasma membrane. GLUT1 was quantitated by Western blot analysis. Experiments were done in duplicate or triplicate, and representative results are shown. GLUT1, at 43 kD, was 5-fold enriched in the Golgi fraction of lactating mammary gland compared to total cellular homogenate. Enrichment continued after 3 h of weaning, but was lost by 5 h of weaning, and diminished further thereafter. Enrichment could be restored by returning the pups to the mother for 5 h , and moreso after 15 h. A second effect of weaning, seen after 10 h, is that total cellular content of GLUT1 begins to decrease. During natural weaning between 18 and 29 postnatal days, a decline in total cellular and Golgi GLUT1 occurs. Unexpectedly, the GLUT1 antibody also identified higher MW proteins. A 72 kD protein showed even more striking Golgi enrichment than GLUT1, and also showed loss of Golgi enrichment during weaning. Intermediate MW forms at 50 and 65 kD were also observed, and it seemed that the degree of GLUT1 glycosylation was an important determinant of its subcellular targeting. An 80 kD protein was interpreted as the major ubiquitin-containing protein present in these fractions, and presumably represented the ubiquitin-conjugated 72 kD protein. An important control, repeating all these experiments using antibody preabsorbed with the antigenic peptide used to generate the GLUT1 antibody, gave no signal. This seemed to prove the specificity of the antibody for all forms identified. Important revisions in the interpretation of these experiments are given below in light of new experiments with peptide-affinity purified antibody. Use of this more specific antibody in an effort to confirm these multiple isoforms has failed to do so, as described below.

We had concentrated on differential display as the method of choice for pursuit of Task II, the identification of novel proteins involved in glucose transport in lactating mammary epithelia. One gene previously identified, LDH, has been further studied, and evidence regarding its induction by lactogenic hormones is provided below. Last year's report included progress with several novel expressed sequence tags identified by this process. These ESTs correspond to genes expressed in response to lactogenic hormones but are actually unlikely

to be specifically related to regulation of glucose transport. Another approach I adopted this year, blue native gel electrophoresis, described below, has been effective in specifically identifying a novel protein-protein interaction related to GLUT1 retention by Golgi. Therefore, because a number of recent papers have appeared pointing out the difficulties and large amount of efforts and resources required to bring differential display analyses to a conclusion, and because of rapid progress of the human genome project information which will facilitate idetification of the current sequences, and because of rapid development of DNA microchip arrays, which offer many advantages over differential display, I have deferred further pursuit of the ESTs at this time. I am continuing to search the databases routinely for homology to the currently identified sequences.

Results and Discussion for Current Reporting Period

Task 1: Description of the developmental and hormonal regulation of glucose transport and lactose biosynthesis in CIT3 cells and in mammary gland

Our studies during the past year have demonstrated a marked heterogeneity in the behavior of conventional Golgi markers during lactation. To augment our previously reported immunofluorescent detection of GLUT1 targeting to Golgi under the influence of lactogenic hormones and to better define the nature of the intracellular targeting, a series of confocal observations were made. Figure 1 confirms that GLUT1 is targeted to Golgi in CIT3 cells grown in secretion medium, which contains prolactin and dexamethasone, but not in growth medium, where plasma membrane targeting is seen. Bodipy-TR ceramide is used to mark the trans-Golgi. No colocalization is seen, indicating that GLUT1 does not target to the trans-Golgi. Figure 2 is an identical experiment, but cells were treated with Brefeldin A, which disrupts Golgi, at 5 ng/ml, for 30 minutes. Note that the trans-Golgi marker is now distributed throughout the cytosol, reflecting the effectiveness of Brefeldin A. In contrast, although GLUT1 targeting is somewhat more punctate and less perinuclear in Figure 2D compared to 1D, it is not scattered throughout the cell. Again, there is no colocalization under these conditions. Figure 3 demonstrates staining for the conventional Golgi markers b-COP, a-mannosidase, and p58 in CIT3 cells grown in secretion medium and in the absence and presence of Brefeldin A. Note that Brefeldin A appears to not completely disrupt targeting of these proteins. B-COP targeting is not affected, a-mannosidase appears to become more perinuclear, and p58 is distributed widely whether or not Brefeldin A is present. Colocalization of GLUT1 with b-COP and a-mannosidase is observed and is enhanced by Brefeldin A. No colocalization of GLUT1 with p58 is seen. We conclude that latogenic hormones cause GLUT1 targeting to cis- and medial but not trans- compartments of Golgi. The results suggest that p58 is not a valid Golgi marker under these conditions.

It was important to test the hypothesis that the Golgi targeted GLUT1 might represent a novel transporter isoform that shares a GLUT1 epitope. This is especially true since the apparent molecular weight of GLUT1 is somewhat higher in cells from secretion medium than in growth medium. Therefore, protein fractions (homogenate; 17000 pellet, enriched in Golgi; 100,000 g pellet, enriched in plasma membrane) from cells were subjected to PNGase F, which removes all sugar residues, and then Western blotted for GLUT1. Figure 4 confirms the difference in molecular weight of GLUT1 under the two conditions. However, after PNGase F digestion, all GLUT1 signal from cells grown in growth medium and secretion medium has an identical apparent molecular weight of about 40 kD, whether from homogenate, Golgi fraction, or plasma membrane fraction. There are no other immunoreactive proteins identified in these experiments, which were carried out with peptide-affinity purifed antibody, in contrast to experiments reported last year. These results are evidence against a novel isoform and also against the idea that glycosylation is important in glucose transporter targeting in mammary gland. Note that this contradicts conclusions drawn in last year's report.

A major focus during this year has been the characterization of developmental changes in GLUT1. Immunocytochemistry and immunofluorescence (Figures 5 and 6, respectively) demonstrate that GLUT1 levels in mouse mammary gland rise during pregnancy and peak during lactation, that GLUT1 targeting is restricted to basolateral and not apical plasma membrane domains, and that intracellular targeting peaks during lactation. The results also remind us that the virgin gland consists primarily not of mammary epithelial cells but of fat cells, which must be kept in mind in interpreting results of subcellular fractionation. In fact, the few virgin mammary epithelial cells seen do express GLUT1. The distribution of GLUT1 by subcellular fraction in lactating gland is shown in Figure 7, indicating an approximately 10-fold enrichment of Golgi compared to homogenate and also a much higher enrichhment than is seen in the plasma membrane fraction. As Figure 8 indicates, this represents an increase in total mammary gland GLUT1 from very low levels in virgin gland, where GLUT1 is targeted to plasma membrane. By late pregnancy, GLUT1 levels have risen, and there is substantial targeting to the Golgi pellet, although Figure 8 indicates that density gradient analysis does not support the localization of this GLUT1 to Golgi, but rather to high density compartments. In contrast, the even higher levels of GLUT1 seen during lactation and predominantly enriching the 17,000 g pellet are predominantly in the low-density Golgi fractions (Figure 8). The forced weaning experiment shown in Figure 8 has also been repeated since last year, again with no detection of higher MW proteins. The conclusion from last year's report that the Golgi targeting of GLUT1 can change rapidly and reversibly still stands; in fact, Figure 8 demonstrates that the process is even more rapid than previously appreciated, with a half-time of only about 2 hours.

We have sought to demonstrate the validity of the fractionation scheme by measuring levels of marker enzyme protein and activity. Generally, the results have shown that GLUT1 itself seems to be a better Golgi marker than the conventional Golgi markers, which seem to have a broader and more heterogeneous distribution. For example, Figure 10 demonstrates that the Golgi marker galactosyltransferase is enriched in the Golgi fraction, but the enrichment is only 2-fold compared to homogenate and 6-fold compared to plasma membrane. Similarly, b-COP is broadly distributed among subcellular fractions (Figure 11), although its Golgi targeting demonstrates the same pattern as GLUT1 during forced weaning during the first five hours. Interestingly, just as for GLUT1, an increase in signal in the 17000 g pellet is seen at 10 h; for neither protein have we yet blotted the density gradient to determine whether this represents targeting to low-density or to higher density fractions. Clearly, at 1.5h after weaning, the b-COP signal of the 17000 g pellet doesreflect Golgi targeting (Figure 12). Interestingly, p58 at no point appears to be a Golgi marker (Figure 13), consistent with the immunocytochemical study (Figure 3). However, a small amount of p58 is detected in the iodixanol density gradient low density fractions (Figure 14). The validity of the plasmamembrane fraction is demonstrated by the high degree of enrichment by alkaline phosphatase at all timepoints of the forced weaning experiment (Figure 15). The general conclusion from these marker studies is that our procedures reliably produce appropriately enriched fractions. Interestingly, changes seen on forced weaning, particularly with b-COP, suggest that changes in GLUT1 targeting during this time may not be regulated specifically at the level of glucose transport, but more broadly at the level of the Golgi itself, which may be undergoing a dynamic reorganization affecting all of its constituents.

Task 2. Identification of novel proteins involved in glucose transport in lactating mammary epithelia

A major thrust during the first years of this work was the use of differential display to identify genes expressed under stimulation by prolactin and hydrocortisone. This non-targeted strategy was expected to identify a variety of genes, most with no relation to glucose transport. We have previously reported that LDH was one of the genes identified by this analysis. Figure 16 confirms that LDH activity is indeed induced approximately 2.5-fold by prolactin and hydrocortisone. I am still considering the significance of this; I am planning to study mouse mammary gland. As discussed above, I have made a strategic decision to not further pursue certain apparently novel genes at this time. In addition to the reasons provided above, this also reflects success with more targeted approaches described below.

The first of these is the successful construction of an expression vector for a GLUT1-EBFP (enhanced blue fluorescent protein) fusion protein, and the expression of this fusion protein by liposome-mediated transfection of CIT3 cells

(Figure 17). Note that in these cells, GLUT1 appears to be intracellular; although they are grown in the absence of lactogenic hormones. We will need to reproduce this result in other clones and to characterize the subcellular compartments involved in order to test whether these cells will provide a good model. We are currently expanding stably transfected clones, which we hope will enable us to study glucose transporter targeting in living cells. We intend to use microinjection as well as other methods to introduce agents that specifically affect GLUT1 targeting. For example, the microinjection of peptides identical to putative Golgi targeting regions of GLUT1 may disrupt GLUT1 targeting, thus identifying specific amino acid sequences of GLUT1 as sites of interaction with a targeting mechanism.

Another fascinating and potentially very direct approach has involved the use of dodecyl maltoside and Blue Native protein electrophoresis. This approach is designed to not disrupt associations between proteins; thus, the signal on the gel reflects the sum of the molecular weight of the protein being detected and the molecular weight of other protein(s) associated with it. When this procedure is carried out on iodixanol density gradient purified Golgi fractions form lactating mammary gland (Figure 18) or fromCIT3 cells grown in secretion medium (data not shown), a single broad band running at about 130 kD is identified. The simplest interpretation of this would be that GLUT1 is associated with a single protein of about 80-90 kD. This would presumably be a Golgi resident protein responsible for retaining GLUT1 in the Golgi. Note that virtually no signal is identified at about 45 kD, where monomeric GLUT1 would be seen; thus, under these conditions, virtually no Golgi GLUT1 exists as a free integral membrane protein. Our next steps are to further purify this protein by denaturing gel electrophoresis and by 2-D gel electrophoresis, to obtain peptide sequence information, and to identify the associated protein as a known protein or to clone it. This protein may provide a necessary and sufficient mechanism for Golgi targeting of GLUT1, obviating the pursuit of less targeted approaches such as differential display.

Task 3. Examination of a possible association between abnormal glucose transport and the neoplastic phenotype

I chose to begin our studies of neoplastic cells with well established cultured cell lines, so we could develop an understanding of the mechanisms of glucose transport in neoplastic mammary epithelia before utilizing human tissue. These experiments were generally designed to test the hypothesis that neoplastic mammary epithelial cells would demonstrate high levels of glucose trnasport activity and GLUT1 protein to support a high rate of glucose utilization, and that GLUT1 in these cells would be targeted primarily to the plasma membrane, again in order to support a high rate of glucose utilization. The hope I set forth in my proposal was that activation of the Golgi targeting mechanism for GLUT1

in a neoplastic cell might serve to deprive the cell of substrate, limit its growth, and make it more vulnerable to chemotherapy.

Initially MCF7 cells, a non-metastatic line, and MDA231 cells, a metastatic line, were chosen. 2-Deoxyglucose uptake in these cells is compared to CIT3 cells in Figure 19. Surprisingly, MCF7 cells showed very low glucose uptake and were not responsive to prolactin and hydrocortisone. In contrast, MDA231 appeared to demonstrate an exaggeration of the pattern seen in CIT3 cells, with a very high glucose uptake under basal condition, and 73% inhibition by prolactin and hydrocortisone. The dependence of the inhibition of glucose transport activity on a synergism between prolactin and hydrocortisone is shown in Figure 20. Taken together, these results suggested very high total cellular levels of GLUT1 with highly regulated changes in subcellular distribution paralleling those seen in CIT3 cells.

Results of subcellular fractionation could not have differed more from what was predicted. In CIT3 cells, lactogenic hormones cause a strong induction of GLUT1 in the homogenate fraction and a shift from plasma membrane to Golgi targeting (Figure 21, left panel). In contrast, hormonal treatment affects neither the amount nor the subcellular targeting of GLUT1 in MDA231 cells. Most surprising of all in these cells with a high glucose transport activity in basal medium, is that there is very little GLUT1 detected in the homogenate and plasma membrane fractions. This strongly suggests that GLUT1 is not the glucose transporter responsible for the very high rates of glucose transport activity in MDA231 cells. We do plan to attempt to identify what must be a novel glucose transporter or an "oncotransporter" that must account for glucose uptake in these cells.

Immunofluorescent microscopy confirmed this interpretation. In both growth medium (data not shown) and in secretion medium (Figure 22), GLUT1 was found exclusively in a very atypical intracellular compartment composed of heterogeneous but frequently relatively large spheres. This resembles a pattern seen in some cells for lysosomes or peroxisomes. We do not yet have any marker data to indicate whether these are atypical Golgi or other organelles. There is no plasma membrane staining of GLUT1. We are considering the possibility that these tumor cells may have a very high activity of the machinery that normally targets GLUT1 intracellularly during lactation, and that this is expressed abnormally even when cells are not exposed to lactogenic hormones.

Finally, again in an effort to understand transporter targeting in cells before undertaking work with human specimens, we have begun working with human mammary epithelial cells isolated from milk during the first week of lactation (Figure 23). These cells target GLUT1 similarly to other normal cells we have examined; note the similarity between Figure 23 (human) and Figure 1F (mouse). We plan to thoroughly characterize glucose transport activity and regulation in these cells and in other established tumor cell lines before beginning work with human tumor specimens.

Discussion in Relation to Statement of Work

I have referred several times during the course of the report to the SOW, and I structured the Body section according to the SOW. In summary, with respect to Task 1, this task is substantially complete except for studies of lactose biosynthesis which are ongoing and which I have not reported on. For Task 2, efforts are now very focused on what are likely to be productive strategies, as identification of a potential 80-90 kD protein associated with Golgi GLUT1 appears to be a breakthrough that will obviate the need for non-specific strategies such as differential display. With respect to Task 3, which specifically addresses breast cancer, we now have evidence that a breast cancer cell line exhibits very unusual glucose transport properties, including abnormal targeting of the GLUT1 glucose transporter and the apparent expression of a novel or "oncotransporter." We are characterizing this further before proceeding with studies of human tissues and mammary gland transplantation. I foresee no difficulty in completing all tasks on the SOW during the final year of my New Investigator Award.

Conclusions

1. In normal CIT3 mammary epithelial cells, GLUT1 colocalizes with Golgi markers b-COP and a-mannosidase but not with the trans-Golgi marker Bodipy TR-ceramide.
2. GLUT1 targeting to Golgi is sensitive to Brefeldin A.
3. There are no higher molecular weight isoforms of GLUT1.
4. Glycosylation plays no role in GLUT1 targeting to Golgi.
5. There is no evidence that lactogenic hormones stimulate expression of a novel glucose transporter.
6. Forced weaning disrupts targeting of GLUT1 to Golgi even more rapidly than reported last year.
7. Golgi markers demonstrate heterogeneity in subcellular distribution in mammary gland.
8. Changes in Golgi markers with forced weaning suggest that changes in GLUT1 targeting during that time may reflect a dynamic reorganization process affecting all Golgi constituents.
9. LDH is differentially expressed in response to prolactin and hydrocortisone.
10. GLUT1-EBFP fusion protein offers the opportunity to study transporter targeting in living cells.
11. Golgi GLUT1 purified under non-denaturing conditions has an apparent molecular weight of 130 kD, suggesting that it may be associated with a protein of 70-90 kD.
12. MCF7 cancer cells exhibit very low rates of glucose transport.
13. MDA231 cells exhibit very high rates of glucose transport but do not appear to utilize GLUT1 for this purpose, suggesting expression of a novel transporter or "oncotransporter."
14. MDA231 cells sequester GLUT1 in an atypical-appearing intracellular compartment whether or not prolactin and hydrocortisone are present.
15. GLUT1 targeting in human mammary epithelial cells isolated from milk parallels findings in mouse cells.

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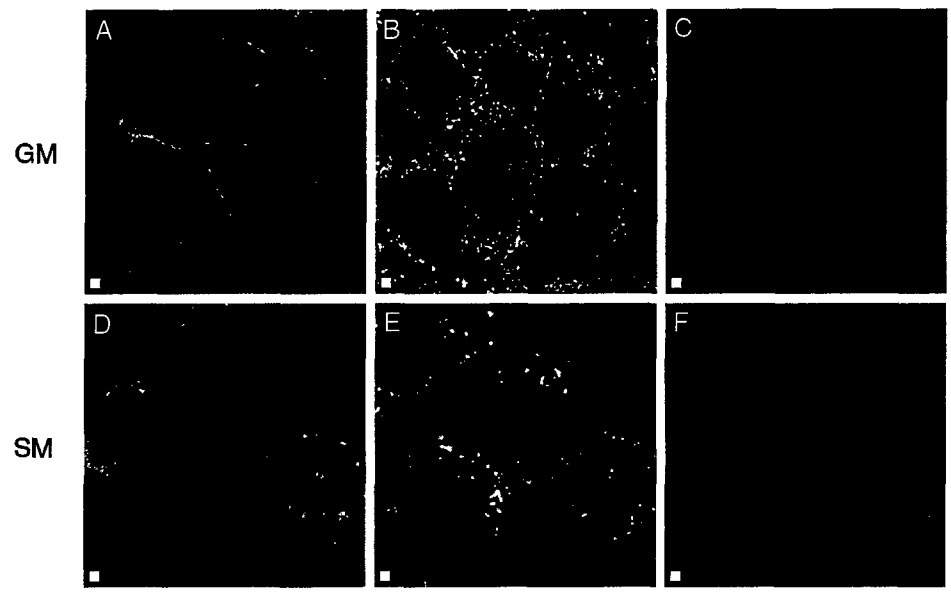

Figure 1. Confocal immunofluorescent microscopy of CIT3 mouse mammary epithelial cells, carried out as described in the text, demonstrates GLUT1 (green) staining plasma membrane in growth medium (A), but staining intracellular compartments in secretion medium (D), which contains prolactin and hydrocortisone. Bodipy-TR ceramide stains trans-Golgi (B,E) red. Colocalization would be indicated by yellow signal in C and F. Note that there is no colocalization of GLUT1 and the trans-Golgi marker.

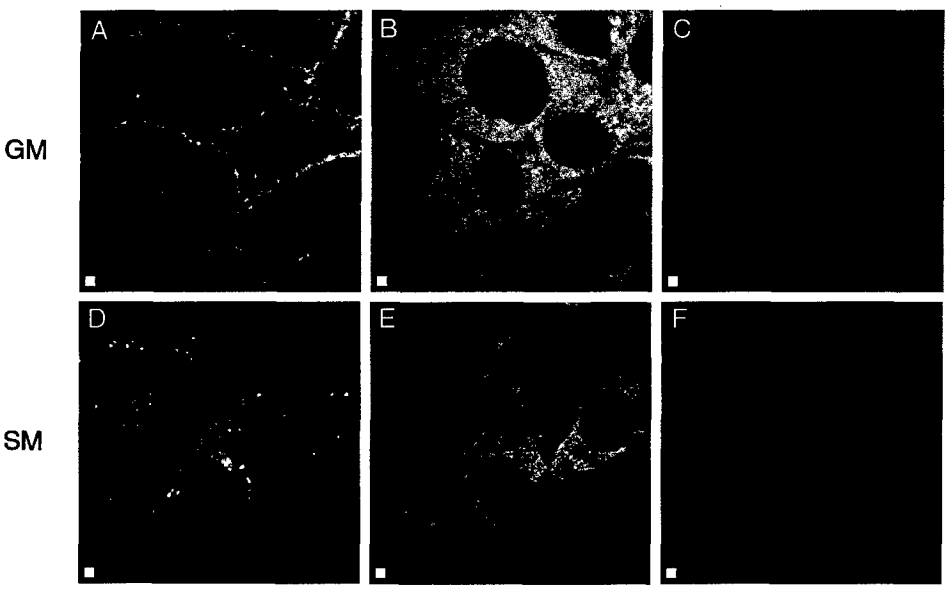

Figure 2. Confocal immunofluorescent microscopy of CIT3 mouse mammary epithelial cells exposed to Brefeldin A, which causes dissociation of Golgi, carried out as described in the text. This demonstrates GLUT1 (green) staining plasma membrane in growth medium (A), but staining intracellular compartments in secretion medium (D), which contains prolactin and hydrocortisone. Bodipy-TR ceramide stains trans-Golgi red (B,E) ; note the difference in its staining compared to Figure 1, reflecting Brefeldin A's effect. Note that there is no colocalization of GLUT1 and the trans-Golgi marker in secretion medium. Note that the GLUT1 staining in secretion medium has a punctate quality compared to Figure 1D and F, where Brefeldin A was absent.

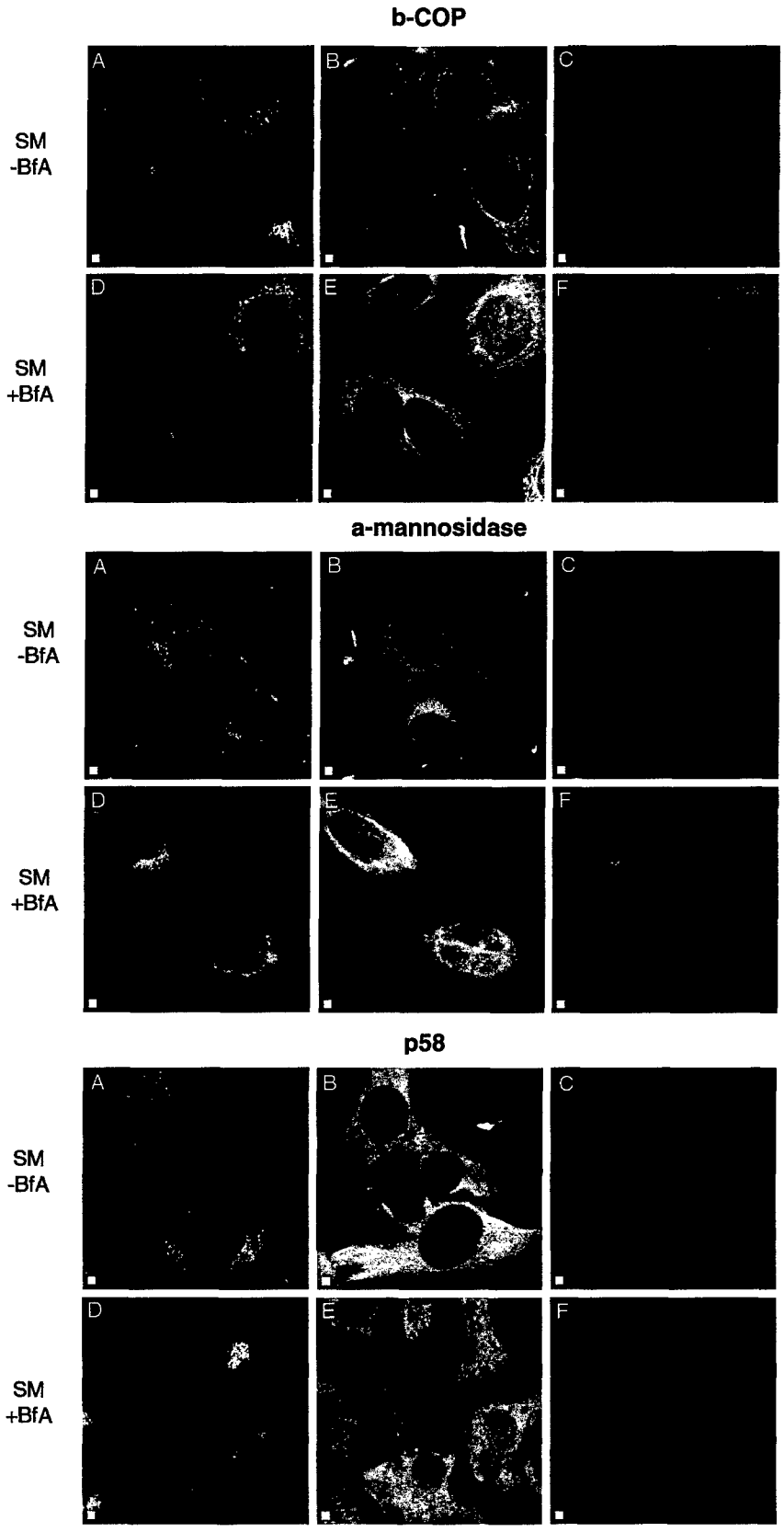

Figure 3. Confocal immunofluorescent microscopy of CIT3 mouse mammary epithelial cells in the absence and presence of Brefeldin A (BfA), which causes dissociation of Golgi, carried out as described in the text. All cells were grown in secretion medium, which contains prolactin and hydrocortisone. Conventional Golgi markers b-COP (upper panels), a-mannosidase (medium panels), and p58 (lower panels) are shown in B and E of each set, representing the absence and presence of BfA, respectively. Yellow signal in C and F for b-COP and a-mannosidase reflects colocalization of these proteins with GLUT1. There is no evidence of colocalization for GLUT1 and p58.

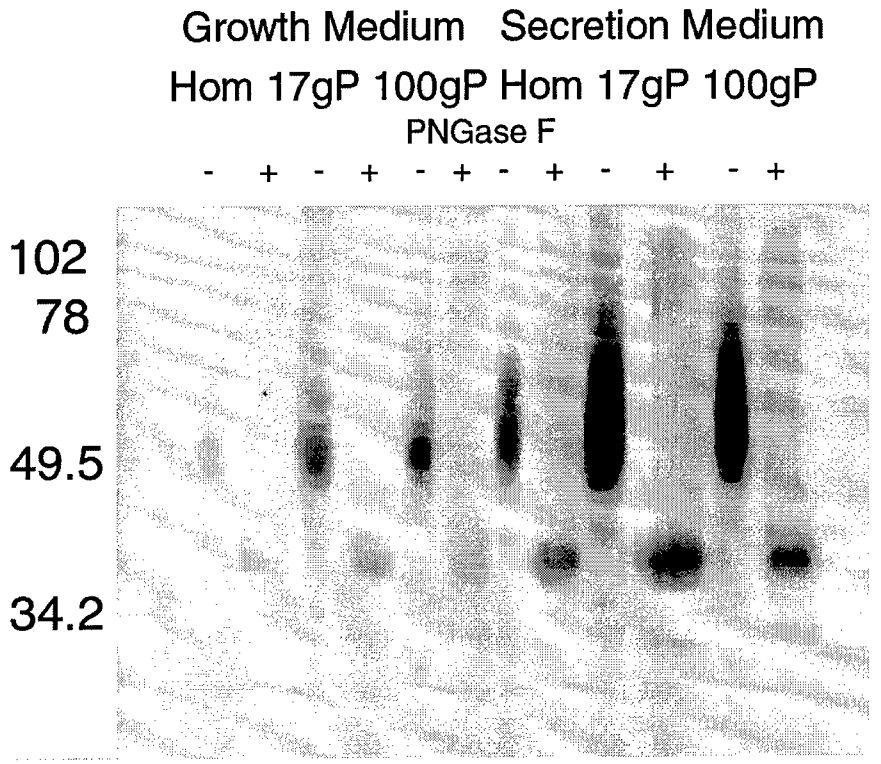

Figure 4. Western blotting for GLUT1 of homogenate, 17,000 g pellet (17gP), and 100,000 g pellet (100gP) fractions containing equal amounts of protein from CIT3 cells from growth medium (left) and secretion medium. (right). Fractions were treated with PNGase F (+) to remove sugar residues; untreated samples (-) are also shown. Note the several-fold induction of GLUT1 in secretion medium compared to growth medium, and a shift in GLUT1 signal from the 100,000 g pellet, which contains plasma membrane, of cells in growth medium, to the 17,000 g pellet, which contains Golgi, in cells grown in secretion medium. Note the identical molecular weights of deglycosylated GLUT1 from cells in growth medium and secretion medium, suggesting that no new isoform is induced in secretion medium. Note also that GLUT1 is somewhat more highly glycosylated in secretion medium, but that glycosylation has no effect on subcellular localization.

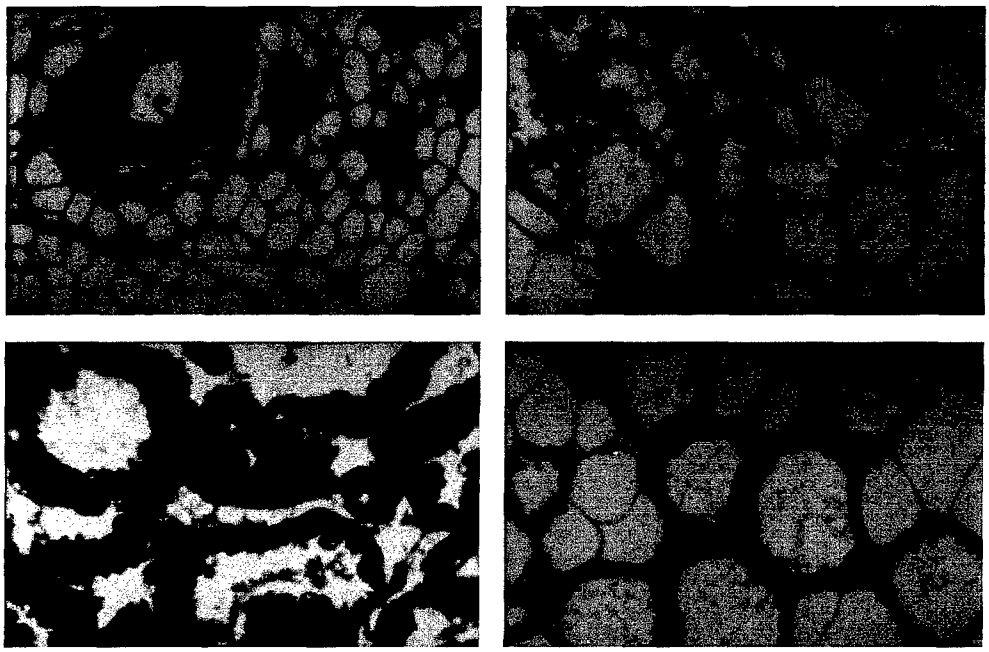

Figure 5. Immunocytochemistry for GLUT1 using alkaline phosphatase secondary antibody in mammary gland from virgin (upper left), day 20 pregnant (upper right), lactating day 18 (lower left), and involuting day 21 (lower right). GLUT1 is stained in brown; the sections are also stained with hematoxylin and eosin according to standard protocols. Note the basolateral and not apical staining of GLUT1. Note also that the virgin gland consists primarily of fat, with relatively few epithelial cells.

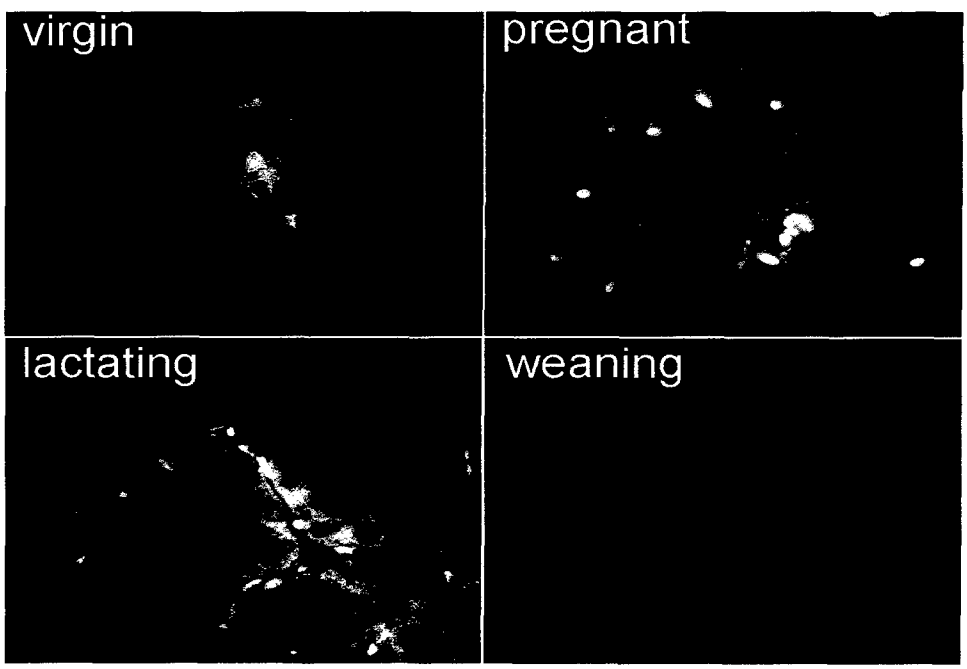

Figure 6. Immunofluorescence using GLUT1 primary antibody and fluorescent secondary antibody in mammary gland from virgin (upper left), day 20 pregnant (upper right), lactating day 18 (lower left), and involuting day 21 (lower right). Note the basolateral staining of GLUT1; as above, virtually no GLUT1 is detected in the apical membrane of the mammary epithelial cells. Note that this method is better suited to detection of intracellular signal. As in Figure 5, signal intensity falls significantly between days 18 and 21 of lactation.

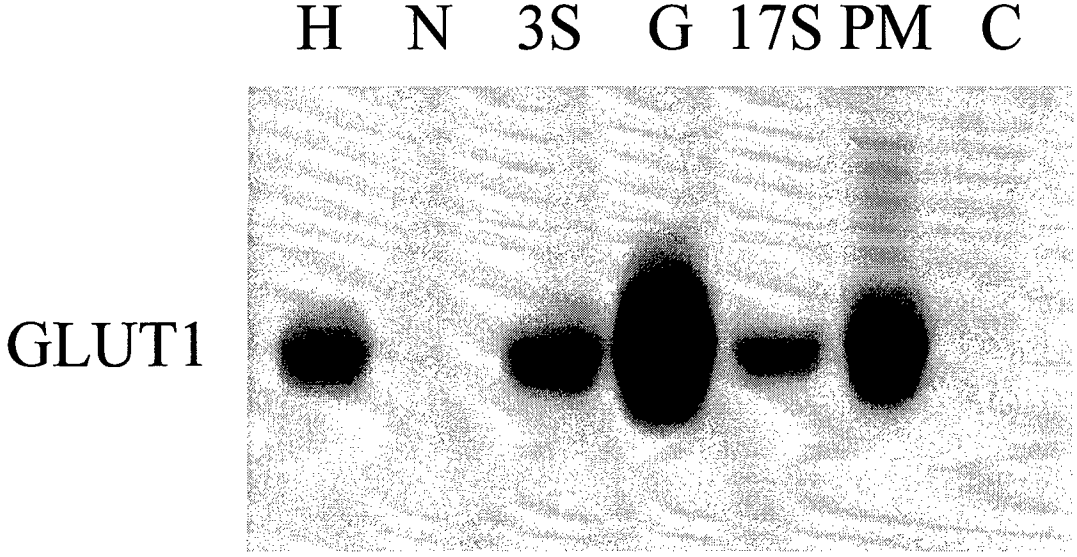

Figure 7. Western blotting of subcellular fractions of day 18 lactating mouse mammary gland. Shown are equal amounts of protein from: H- homogenate, reflecting total cellular levels, N- 3000 g pellet, reflecting nucleus, S- 3000 g supernatant, G- 17000 g pellet, including Golgi, PM- 100000 g pellet, including plasma membrane, and C- 100000 g supernatant, reflecting cytosol and non-sedimenting vesicles. Note that targeting to the 17000 g pellet, which reflects Golgi targeting, dominates during lactation. Note also the absence of any other signals detected by the GLUT1 antibody. This is in contrast to last year's report and demonstrates the artifactual nature of bands previously noted at 80, 72, 65, and 50 kD.

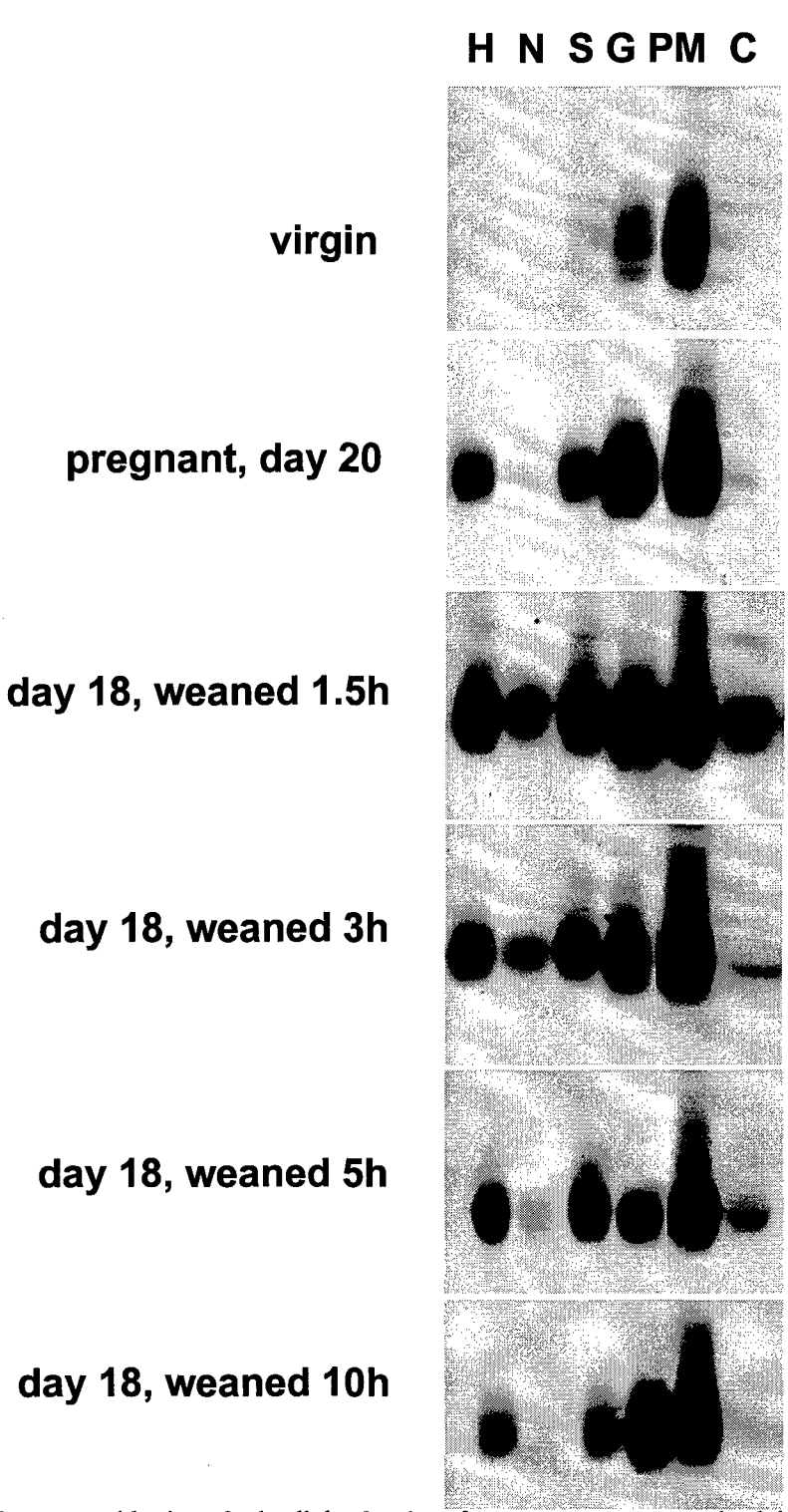

Figure 8. Western blotting of subcellular fractions from mouse mammary gland during development and lactation, and during forced weaning. Shown are equal amounts of protein from: H- homogenate, reflecting total cellular levels, N- 3000 g pellet, reflecting nucleus, S- 3000 g supernatant, G- 17000 g pellet, including Golgi, PM- 100000 g pellet, including plasma membrane, and C- 100000 g supernatant, reflecting cytosol and non-sedimenting vesicles. Mice were studied as virgins, on day 20 of pregnancy, and on day 18 of lactation, after weaning for 1.5h, 3h, 5h, and 10h. Total cellular content of GLUT1 increases many-fold during pregnancy and lactation, and GLUT1 is redistributed from the plasma membrane to the Golgi. Note that the total cellular level of GLUT1 declines during premature weaning. As in Figure 7 the absence of signals except at the 40-50 kD molecular weight at which GLUT1 is seen.

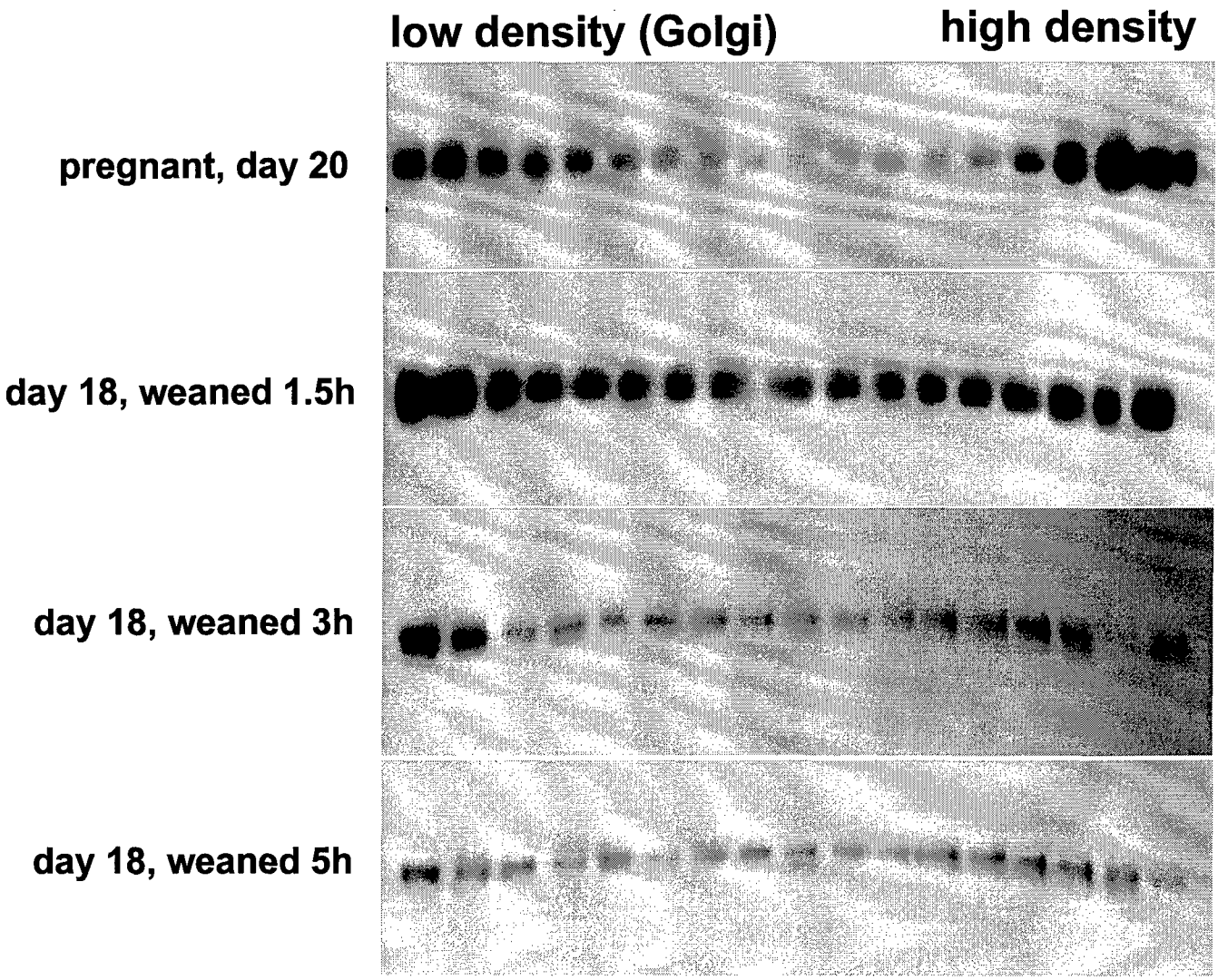

Figure 9. Western blotting using antibody to GLUT1 of iodixanol density gradients from 17,000 g pellets of day 20 pregnant mice and from lactating mice forced to wean for 1.5 h, 3 h, and 5 h. Procedures were carried out as described in the text. Note that the predominant GLUT1 signal in the late pregnant mouse arises not from low-density Golgi fraction, but from higher density fractions. Note also that a decline in Golgi targeting of GLUT1 is evident by 3 h and is pronounced by 5 h. This indicates that forced weaning causes rapid retargeting of GLUT1 away from Golgi. Again, note the absence of signal at other molecular weights.

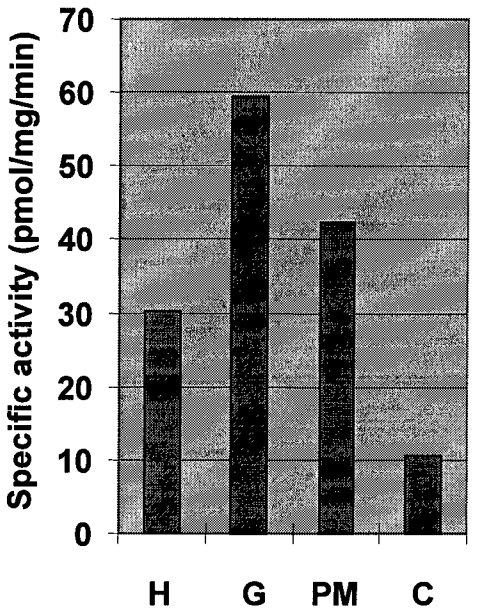

Figure 10. Specific activity of galactosyl transferase, a Golgi marker. Note that galactosyltransferase, assayed according to standard techniques, was enriched in Golgi (G) compared to homogenate (H), plasma membrane (PM), and cytosol (C).

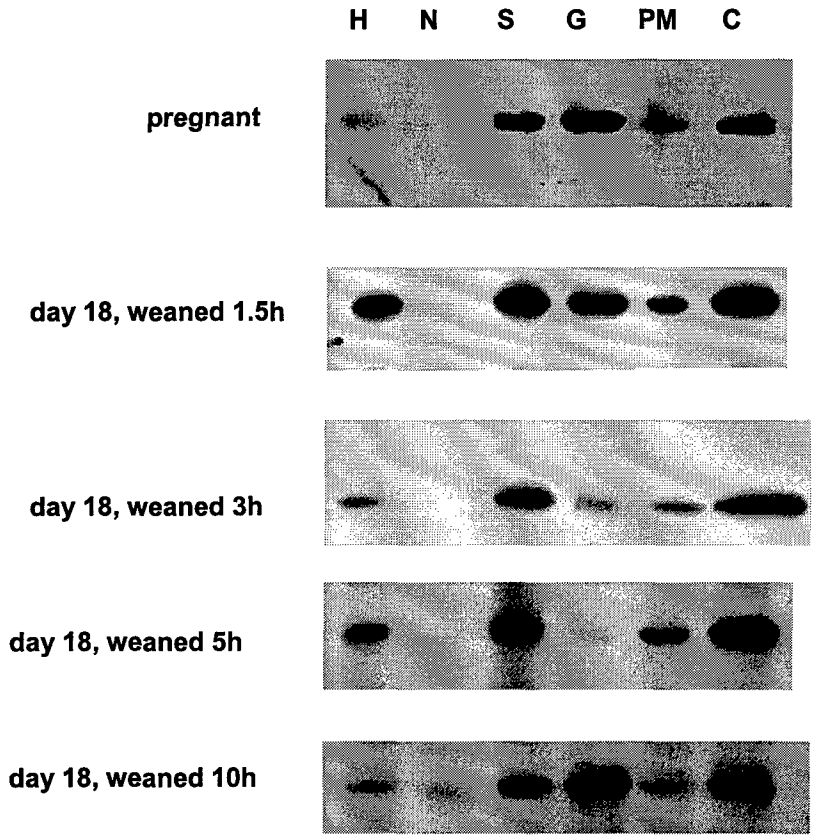

Figure 11. Western blotting for b-COP in mammary glands of day 20 pregnant mice and in mice undergoing forced weaning. In the top panel are samples from a day 20 pregnant mother. The remaining samples are from the mother of 18-day old pups which have been weaned for the time period shown. Shown are homogenate, 3000 g pellet, 3000 g supernatant, 17000 g pellet, 100000 g pellet, and 100000 g supernatant. Equal amounts of protein from each fraction have been subjected to SDS-PAGE . Between 1.5 and 5 h after weaning, the signal in the 17000 g pellet declines, but is again seen at 10h after weaning. Recall that this parallels the reappearance of GLUT1 in the pellet at 10h of forced weaning. Signal in the 100000 g pellet declines over the same time period.

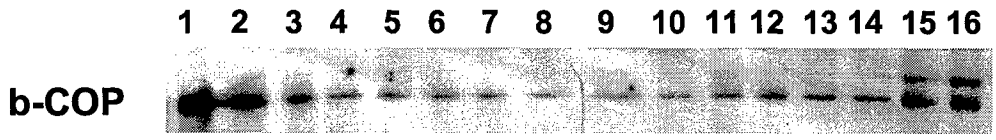

Figure 12. b-COP signal in the 17000 g pellet at 1.5h reflects targeting to Golgi. The 17000 g pellet from the 1.5 h weaned mother was subjected to iodixanol density gradient centrifugation. Lane 1 represents the lowest density fractions, corresponding to Golgi.

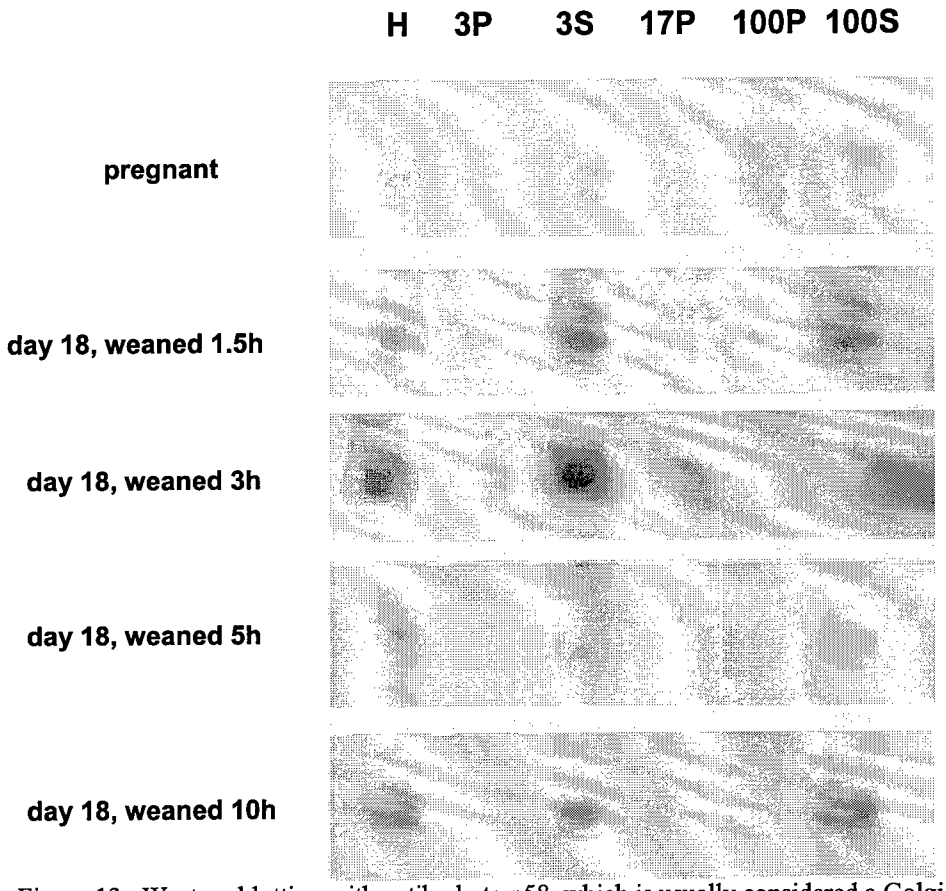

Figure 13. Western blotting with antibody to p58, which is usually considered a Golgi marker In the top panel are samples from a day 20 pregnant mother. The remaining samples are from the mother of 18-day old pups which have been weaned for the time period shown. Shown are homogenate, 3000 g pellet, 3000 g supernatant, 17000 g pellet, 100000 g pellet, and 100000 g supernatant. Equal amounts of protein from each fraction have been subjected to SDS-PAGE. Subcellular localization of p58 in mammary gland does not change during premature weaning. Although p58 is conventionally considered a Golgi marker, little signal is detected in the 17000 g pellet. Signal is primarily detected in the in the 100000 g supernatant at all timepoints.

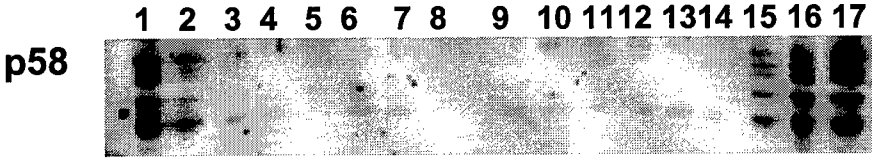

Figure 14. p58 signal in the 17000 g pellet reflects targeting to Golgi and to a high density compartment. The 17000 g pellet from the 1.5 h weaned mother was subjected to iodixanol density gradient centrifugation. Lane 1 represents the lowest density fractions, corresponding to Golgi. The antibody detects five distinct signals from MW 58kD to 42 kD which we have not yet characterized further. These may represent proteolytic fragments.

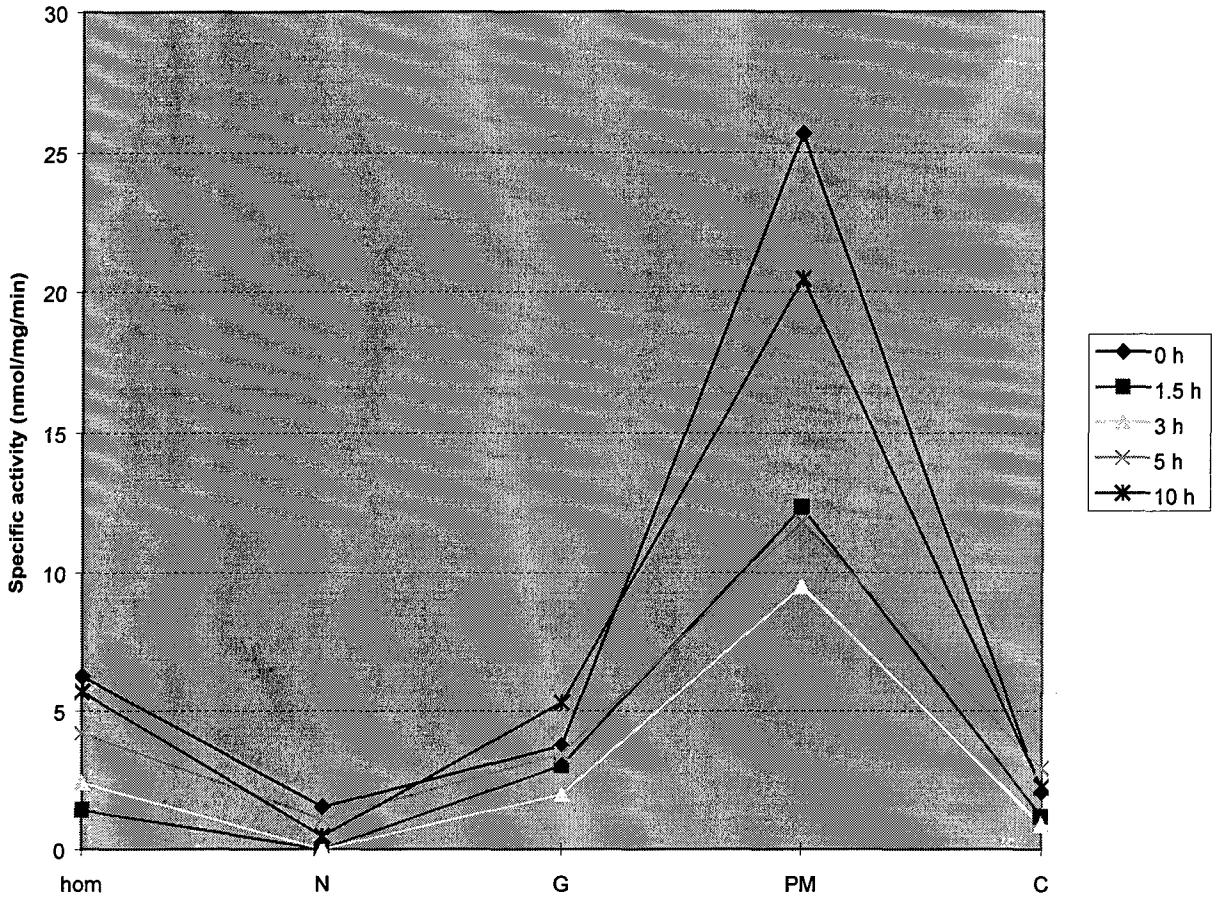

Figure 15. Specific actiivty of alkaline phosphatase, a plasma membrane marker. Alkaline phosphatase activity was assayed according to standard methods and is expressed in mmol/mg/min for homogenate, 3000 g pellet (N), 17,000 g pellet (G), 100,000 g pellet (PM), and 100,000 g supernatant (C). The high degree of enrichment of the PM fraction with this marker at all timepoints (0h, 1.5h, 3h, 5h, and 10h) of a forced weaning experiment confirm the presence of large amounts of plasma membrane in this fraction.

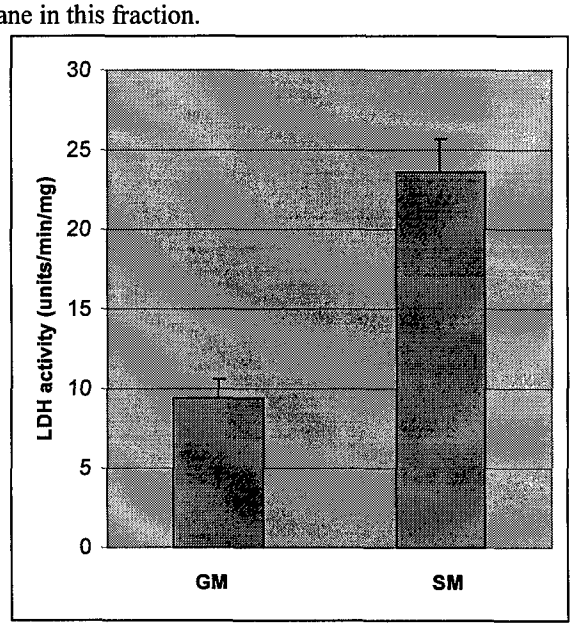

Figure 16. LDH assay of CIT3 cells grown in growth medium and in secretion medium. LDH was assayed in triplicate according to standard assays. The 2.5-fold induction of LDH confirms the identification by diffreential display of LDH as a gene whose expression is stimulated by exposure to lactogenic hormones.

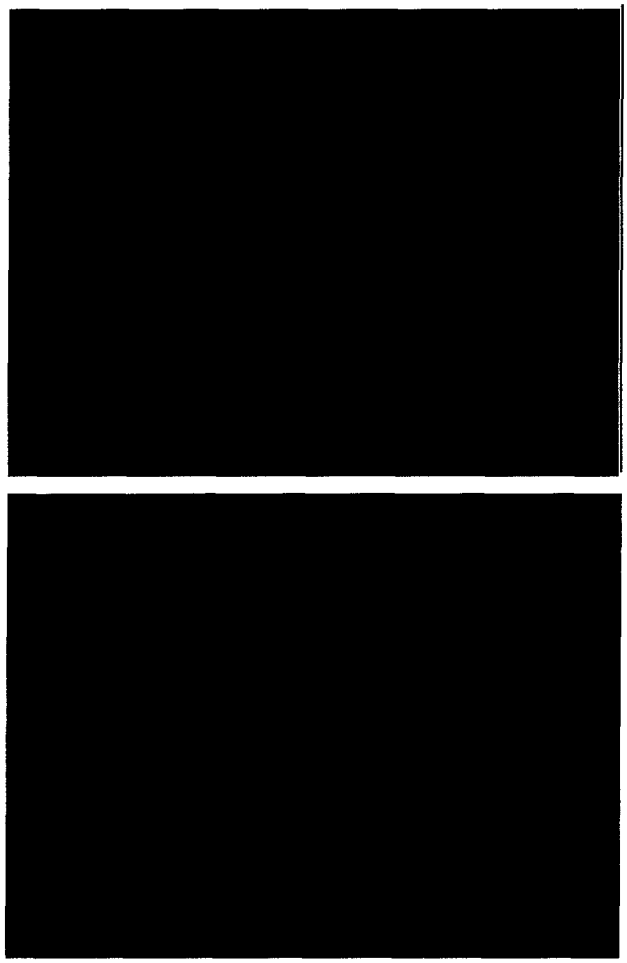

Figure 17. Fluorescence microscopy of stably transfected CIT3 cells expressing a fusion protein consisting of GLUT1 and enhanced blue fluorescent protein (EBFP). Cells were exposed to fluorescence using a NU filter set, 370-380 nm, and images were captured with a black-and-white CCD camera; color was restored in Adobe Photoshop. Cells are shown at low power in the upper panel and at high power in the lower panel. Note the apparent intracellular distribution of the chimeric protein. Cells were grown in growth medium.

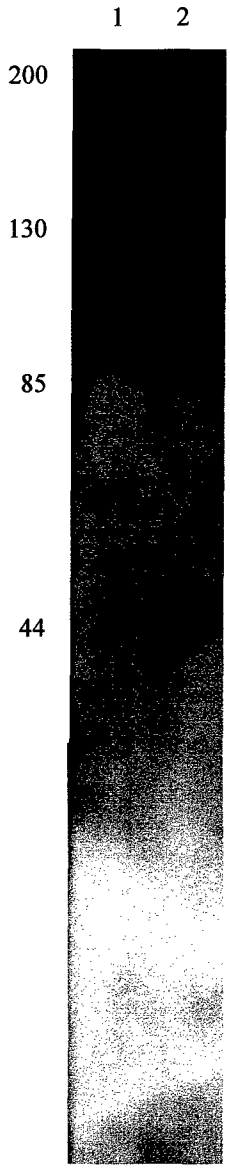

Figure 18. Western blotting with antibody to GLUT1 of a purified Golgi fraction of lactating mammary gland; purification was carried out in the presence of dodecyl maltoside and blue native gel electrophoresis, which involves a non-denaturing gel, was performed. Lanes 1 and 2 are the first and second fractions of an iodixanol density gradient of the 17,000 g pellet from lactating mammary gland. Note a broad signal at about 130 kD, and the virtual absence of signal at 40-50 kD, the molecular weight of monomeric GLUT1. The result indicates that virtually all Golgi GLUT1 is associated with another protein or proteins with a molecular weight of about 80-90 kD.

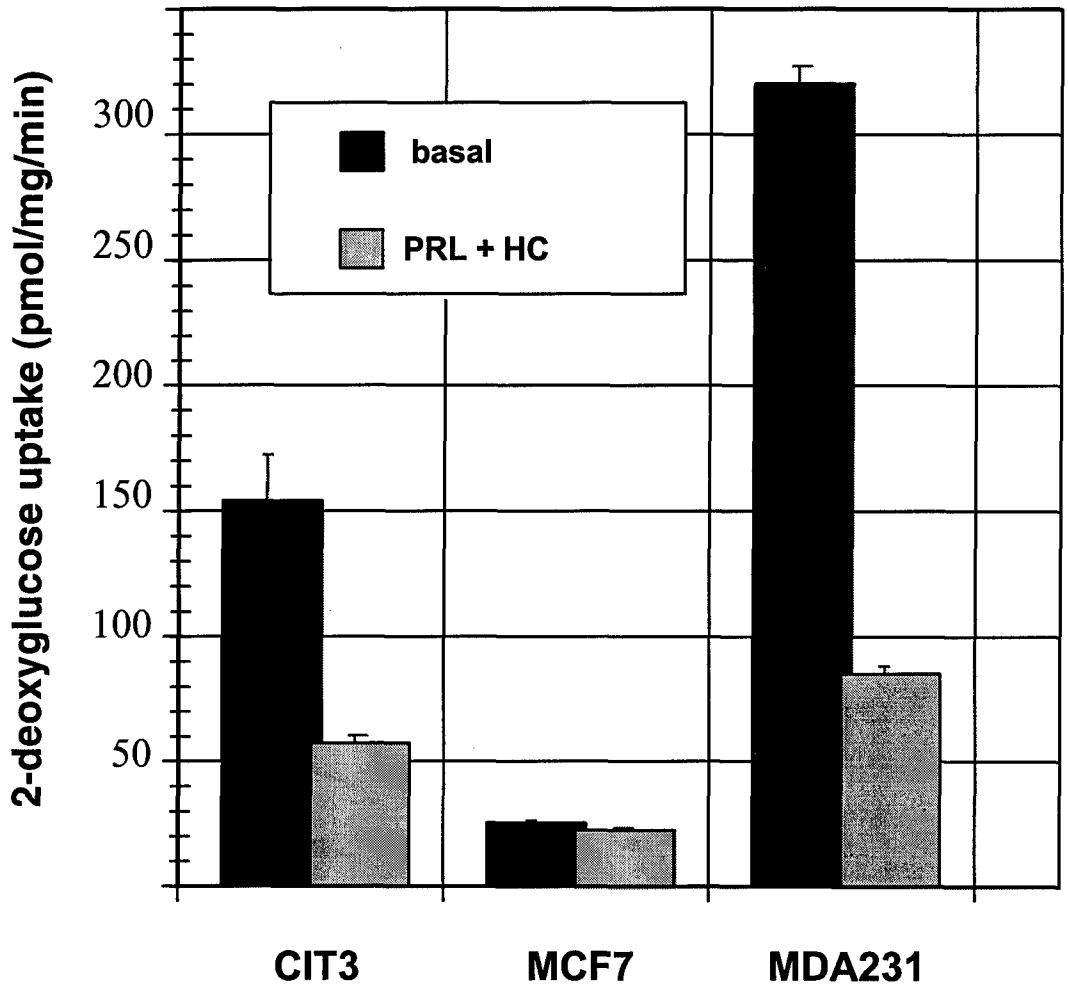

Figure 19. 2-deoxyglucose uptake of normal CIT3 cells, non-metastatic but neoplastic MCF7 cells, and metastatic and neoplastic MDA231 cells. 2-deoxyglucose uptake was measured as described in the text. As we have previously shown, prolactin and hydrocortisone cause intracellular targeting of GLUT1 to Golgi and a decrease in glucose transport activity of CIT3 mouse mammary epithelial cells. However, MCF7 cells have low glucose transport activity and are not affected by prolactin and hydrocortisone. In contrast, MDA231 cells have high glucose transport activity that is 73% inhibited by prolactin and hydrocortisone treatment for four days.

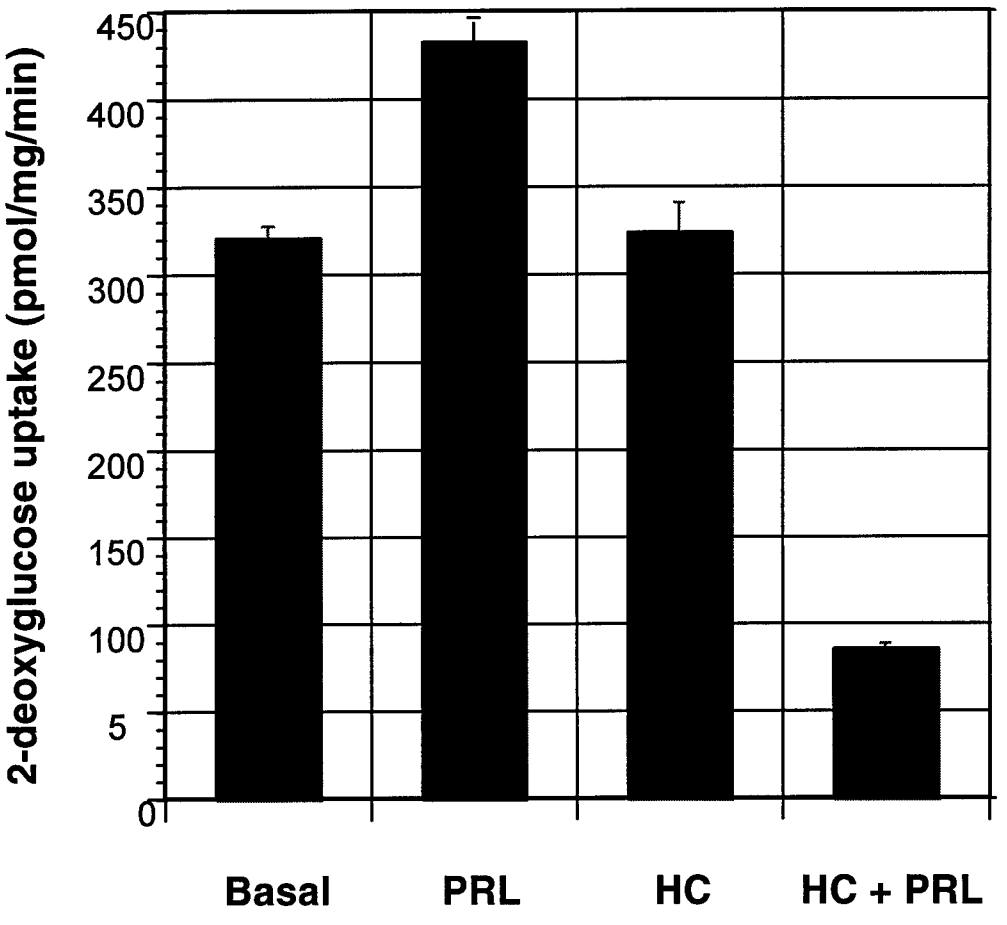

Figure 20. 2-deoxyglucose uptake activity of MDA231 cells exposed to prolactin, hydrocortisone, and both. Measurements were carried out as described in the text. A synergistic effect of the hormones was required to observe any decrease in glucose transport activity.

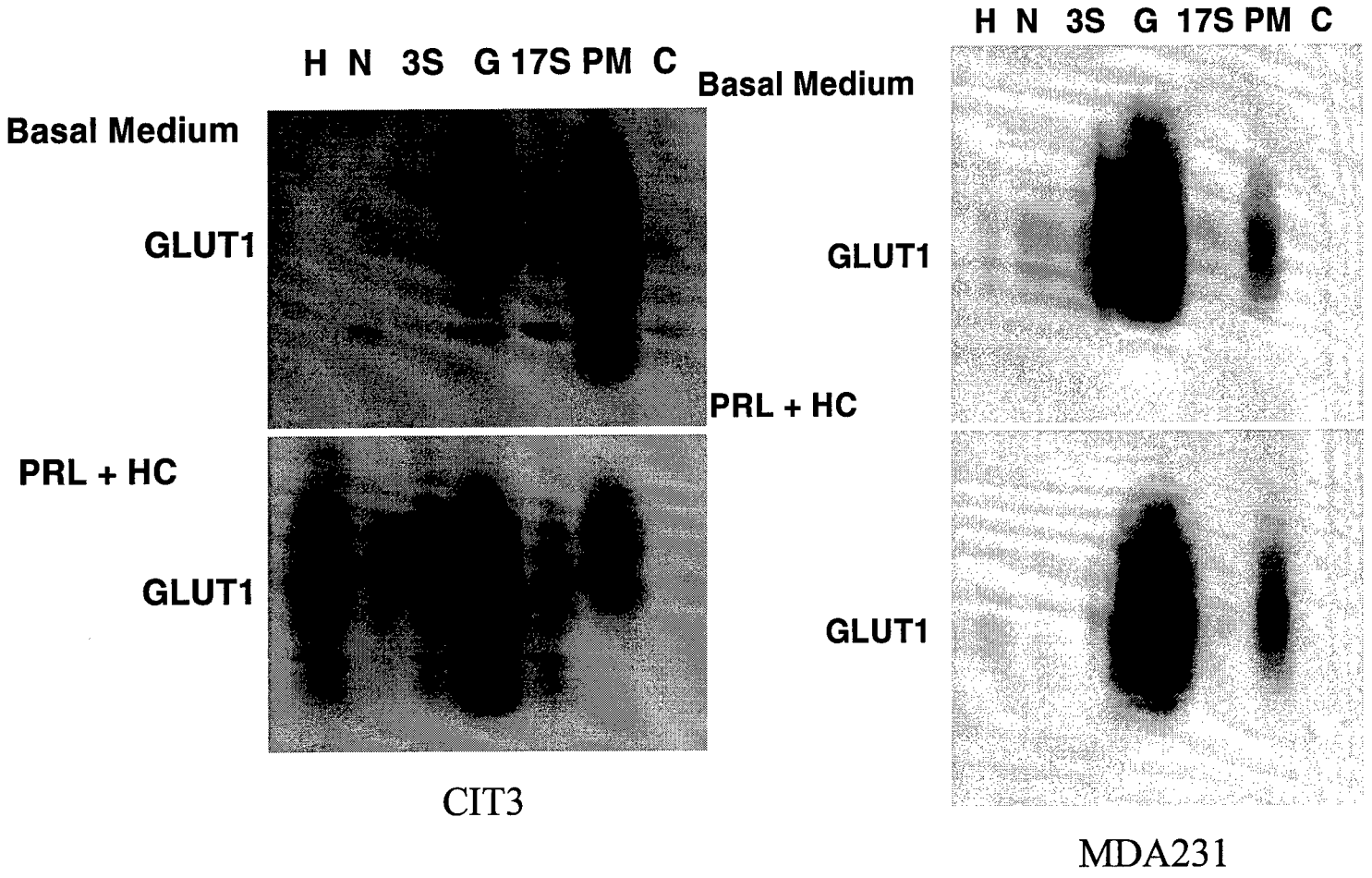

Figure 21. GLUT1 is detected in CIT3 (left panel) and MDA231 (right panel) cells using a highly specific primary antibody against its C-terminus using ECL as described in the text. GLUT1 is localized predominantly to the light mitochondrial (17000 g) pellet, which contains Golgi, in both growth and secretion medium. Plasma membrane markers are found in the microsomal (100000 g) pellet. Lane 1, homogenate; lane 2, 3000g pellet; lane 3, 3000 g supernatant; lane 4, 17000 g pellet; lane 5, 17000 g supernatant; lane 6, 100000 g pellet; lane 7, 100000 g supernatant. The results indicate that these cells have low levels of GLUT1, and that the subcellular distribution of GLUT1 is primarily intracellular and is not affected by hormonal treatment. This is despite the fact that the cells have a high level of glucose transport activity which is 73% inhibitable by hormonal treatment, as demonstrated in the previous figure.

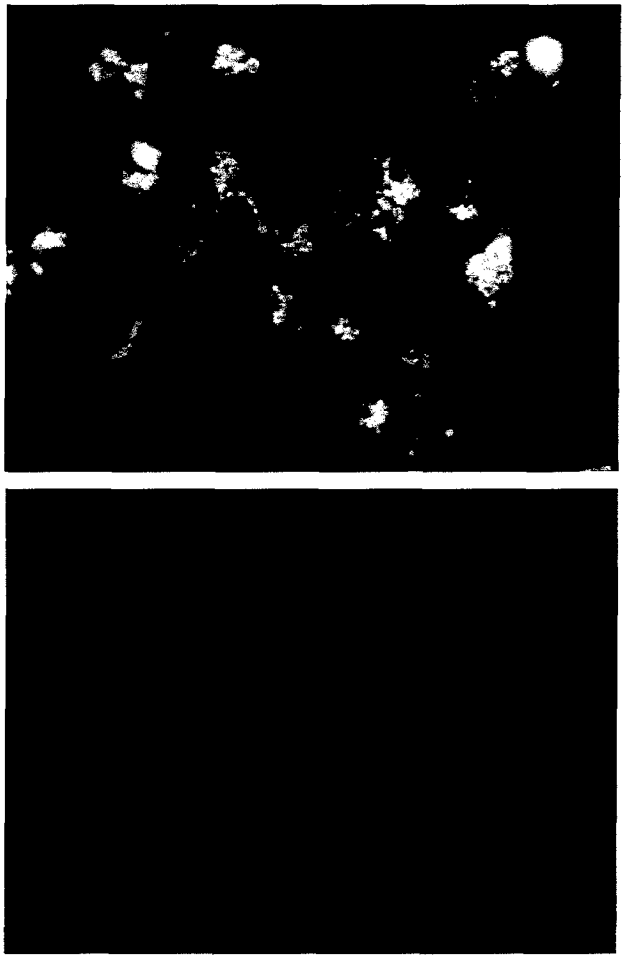

Figure 22. GLUT1 Immunofluorescent staining of MDA231 cells exposed to prolactin and hydrocortisone. The upper panel shows cells exposed to 5 ug/ml of anti-GLUT1; the lower panel is a control showing staining with the irrelevant GLUT4 antibody. Note that GLUT1 is found within the cell in atypical appearing compartments resembling lysosomes or peroxisomes. However, we have not yet stained these cells with Golgi markers, and we can not rule out that this staining is Golgi. Note the absence of GLUT1 in the plasma membrane. The results confirm the subcellular fraactionation studies and strongly suggest that GLUT1 can not be the glucose transporter responsible for the high and regulatable glucose transport activity these cells exhibit.

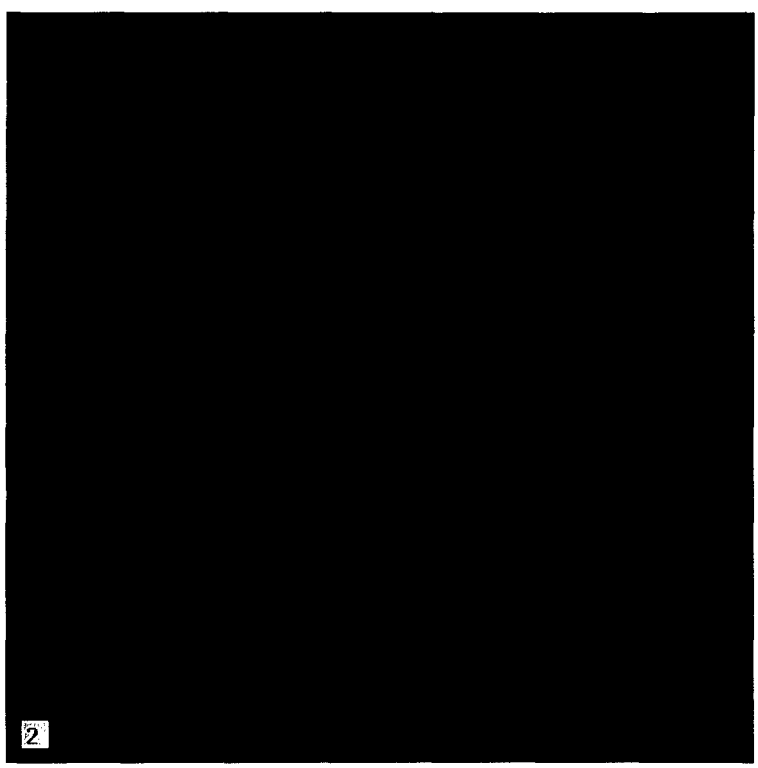

Figure 23. Confocal immunofluorescent microscopy of human mammary epithelial cells isolated from milk during the first week of lactation. Procedures are provided in the text. GLUT1 antibody stains green, while and Bodipy-TR ceramide, a trans-Golgi marker, stains red. Note, just as in CIT3 cells, that there is no colocalization of GLUT1 and the trans-Golgi marker.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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